Functional aspects of fibrinogen β and plasminogen activator inhibitor-1 promoter variants

-Interaction with inflammation and obesity-

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Contents

Chapter 1

General Introduction

CARDIOVASCULAR DISEASE AND THE ROLE OF INFLAMMATION AND HAEMOSTASIS

Cardiovascular diseases are the main cause of death in industrialized countries, and they accounted for 35% of the total mortality in 2001 in the Netherlands [1]. Cardiovascular diseases can be divided into two categories: venous diseases and arterial diseases. Venous diseases include deep venous thrombosis and pulmonary embolism, and arterial diseases include myocardial infarction (MI), peripheral occlusive disease and stroke. An important underlying cause of arterial events is often atherosclerosis, which is a disease of the arterial wall. Atherosclerosis is a progressive disease and may start at an early age. It is characterized by the deposition of cholesterol and minerals in the vessel wall of arteries, resulting in the formation of atherosclerotic plaques. During the progression of atherosclerosis a series of changes to the plaques occurs, in which invasion of inflammatory cells such as macrophages and lymphocytes into the plaque play a key role and weaken the structure of the plaque. Advanced lesions may rupture, which immediately initiates blood coagulation, resulting in the formation of a blood clot or thrombus. If the thrombus blocks the local blood flow, this will severely limit the oxygen supply to the downstream tissue, which then can cause myocardial infarction (MI) or stroke. Several lifestyle factors, such as smoking, nutritional habits and physical activity influence the process of atherosclerosis and other (subsequent) cardiovascular diseases (reviewed by Ross [2] and by Lusis [3]).

Factors that contribute to the development of cardiovascular disease have been investigated in many epidemiological studies. These studies have led to the identification of now well-established cardiovascular risk factors such as smoking, hypertension, elevated plasma cholesterol levels, diabetes, an increased body mass index (BMI) and a low level of physical activity [4,5]. The traditional risk factors that are associated with metabolism (e.g. increased BMI, elevated cholesterol-, triglyceride-, lipoprotein-, insulin- or glucose levels, hypertension) often cluster in an individual, and they are together referred to as the metabolic syndrome (reviewed by Reilly and Rader [6]). Nowadays there is much interest in the metabolic syndrome and in the importance of obesity in particular, as the number of overweight people has reached epidemic proportions in parts of the Western world [7].
The traditional risk factors are important determinants of cardiovascular disease, but they cannot explain the entire risk, indicating that additional factors are also important [8]. These other factors that can contribute to the risk of cardiovascular disease include the plasma levels of blood coagulation factors and inflammatory factors [9-11].
Cardiovascular disease has a genetic component [12,13]. There are many common genetic changes (polymorphisms) that influence the activity or the plasma

levels of proteins important in coagulation, inflammation or metabolism, and these polymorphisms might contribute to the risk of cardiovascular disease. In addition, there is evidence that the effect of genetic variation may be particularly strong in the presence of specific environmental factors (reviewed by Humphries *et al* [14]). Common variations in cardiovascular disease genes under the influence of specific external factors are the subject of this thesis. In addition to the common polymorphisms, rare genetic disorders with severe cardiovascular consequences also exist, but they are beyond the scope of this thesis.

A strong relationship is present between cardiovascular disease and inflammation. Elevated plasma levels of inflammatory factors such as interleukin-6 (IL6), C-reactive protein (CRP) and fibrinogen are associated with an increased risk of atherosclerosis and coronary events [10,11,15-17]. These inflammatory factors (IL6, CRP and fibrinogen are also called acute-phase proteins) play a dual role. On the one hand, their plasma levels reflect the severity of underlying atherosclerosis, which can be regarded as an inflammatory process [2]. On the other hand, these factors may contribute directly to the progression of the disease [18-20].

Blood coagulation is counteracted by fibrinolysis, and the regulation of both processes is strongly interrelated. Blood coagulation and fibrinolysis together are called the haemostatic balance (Figure 1). Blood coagulation is a defense mechanism of the body that stops bleeding, and the main functions of fibrinolysis are to prevent the obstruction of blood vessels and to remove the clot after wound healing. The process of blood coagulation is the result of a cascade of activation of the various clotting factors, leading to the formation of thrombin. Thrombin cleaves the soluble fibrinogen molecule, resulting in the formation of insoluble fibrin fibers, which form the blood clot together with the platelets. Fibrinolysis is the process of clearance of the fibrin clots, and a central step in fibrinolysis is the degradation of fibrin by plasmin. Plasmin is formed after the cleavage of its precursor plasminogen by plasminogen activators, and this process is controlled by plasminogen activator inhibitor-1 (PAI-1). Several studies have shown that the hypercoagulable state resulting from increased plasma levels of factors that promote coagulation or inhibit fibrinolysis are associated with an increased risk of arterial cardiovascular disease (reviewed by Folsom [21]).

Inflammation and coagulation are interconnected in several ways. For instance, inflammation can directly promote blood coagulation by increasing the expression of tissue factor, which initiates the extrinsic coagulation pathway. Another example is the coagulation factor thrombin, which can induce inflammatory responses (reviewed by Esmon [22,23]). These examples illustrate the interrelationship between inflammation, coagulation and cardiovascular disease.

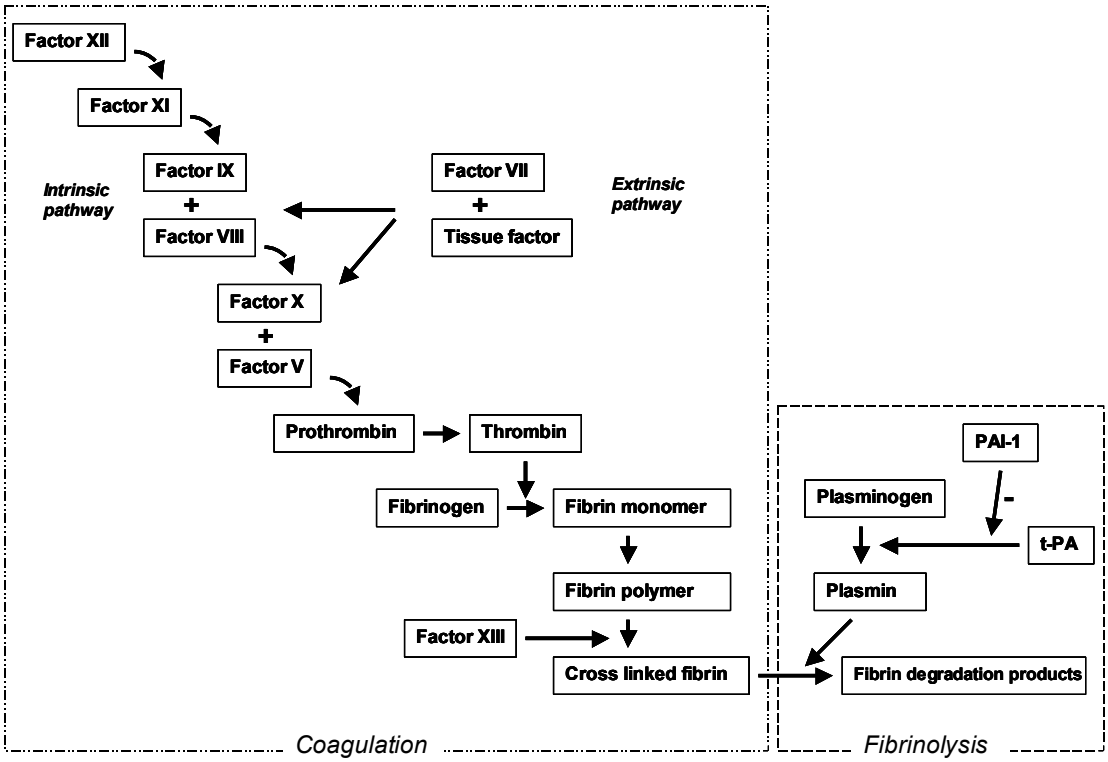

Figure 1: The blood coagulation cascade and the fibrinolytic system. A simplified overview of the haemostatic system is shown. For the sake of clarity, negative and positive feed-back systems have not been indicated.

FIBRINOGEN

THE FIBRINOGEN MOLECULE AND THE FIBRINOGEN GENES

Fibrinogen is a glycoprotein that is present in plasma at concentrations of 2-4 g/l. The liver is the source of fibrinogen. The fibrinogen molecule is composed of two sets of three different polypeptide chains: two Aα chains, two Bβ chains and two γ chains, and the chains are interconnected by disulphide bridges. In the last step of the coagulation cascade, thrombin cleaves off small peptides (the so-called fibrinopeptides A and B) from the amino terminal segments of the fibrinogen Aα and Bβ chains, which turns the soluble fibrinogen molecule into an insoluble fibrin monomer. The fibrin monomers polymerize and form fibrin polymers, which are cross-linked by factor XIIIa, leading to a network of fibrin fibers. Each fibrinogen chain is encoded by a separate gene, and the fibrinogen gene cluster comprises approximately 50 kb and is located on chromosome 4. Transcription of the three fibrinogen chains is tightly coordinated and *in vitro* studies have shown that synthesis of the β chain is rate-limiting in HepG2 hepatoma cells, which is the reason why much emphasis has traditionally been placed on the regulation of β chain gene expression [24].

However, there are indications that elevated α or γ chain production may also increase the assembly of the mature fibrinogen molecule in HepG2 cells [25].

Elevated plasma fibrinogen levels are a strong risk factor for cardiovascular disease [15,26]. Fibrinogen is a coagulation factor, but also an acute phase reactant, and plasma fibrinogen levels strongly increase upon intense inflammatory stimuli such as strenuous exercise, trauma, infection, or surgery [27-29]. In addition, fibrinogen levels are mildly increased in subjects that are exposed to mild and chronic inflammatory stimuli, such as smoking and progressive atherosclerosis [30-32]. As an acute phase reactant, elevated fibrinogen levels are a marker of the severity of the inflammatory process of atherosclerosis, which is an explanation for the association between plasma fibrinogen levels and cardiovascular risk. However, there are also indications that fibrinogen may have a causal role in cardiovascular disease. Fibrinogen is found in the atherosclerotic plaque where it can contribute to the progression of atherosclerosis, for example by increasing the chemotaxis of smooth muscle cells or affecting the stability and structure of the plaque [18,33,34]. In addition, studies with transgenic mice carrying the human apolipoprotein (a) [apo (a)] gene, have also indicated a causal role for fibrinogen in vascular disease. Apo (a) is the protein component of lipoprotein (a), which is a major factor in the development of atherosclerosis. In mice expressing human apo (a) but lacking fibrinogen, the development of atherosclerotic lesions was reduced by 80%, compared to control mice expressing fibrinogen and human apo (a). This again indicates a role for fibrinogen in the generation of atherosclerosis, and suggests that fibrinogen provides a binding site for apo (a) [19].

The main inflammatory inducer of fibrinogen is the cytokine interleukin-6 (IL6). Hepatocytes in culture respond to IL6 administration with a strong increase in fibrinogen production, and plasma IL6 levels are closely correlated with fibrinogen levels *in vivo* [35-38]. The expression of the three fibrinogen genes is coordinately regulated, and the promoter regions of all three fibrinogen genes contain similar regulatory sequences. All three fibrinogen promoters contain IL6 responsive elements (IL6 REs) with the sequence CTGGGA, that have been proven to be functional, but for which the molecular mechanism is still under debate. Both the Aα and Bβ chain promoters contain a binding site for CCAAT-box/enhancer-binding protein (C/EBP, also known as nuclear factor for interleukin-6 expression (NF-IL6) or liver-enriched activating protein (LAP)), and an hepatocyte nuclear factor-1 (HNF-1) element. Finally, in the Bβ and γ chain genes also sequences responsive to glucocorticoids have been identified (Figure 2) [39-43]. In the fibrinogen β gene promoter, the IL6 RE and the C/EBP binding site are both required for the IL6 response of the gene. C/EBPβ has been shown to bind to the C/EBP element, but until now no transcription factors binding to the IL6 RE or to adjacent sequences have been identified, leaving the molecular mechanism of the IL6 response of the fibrinogen β gene partly unexplained [40,44].

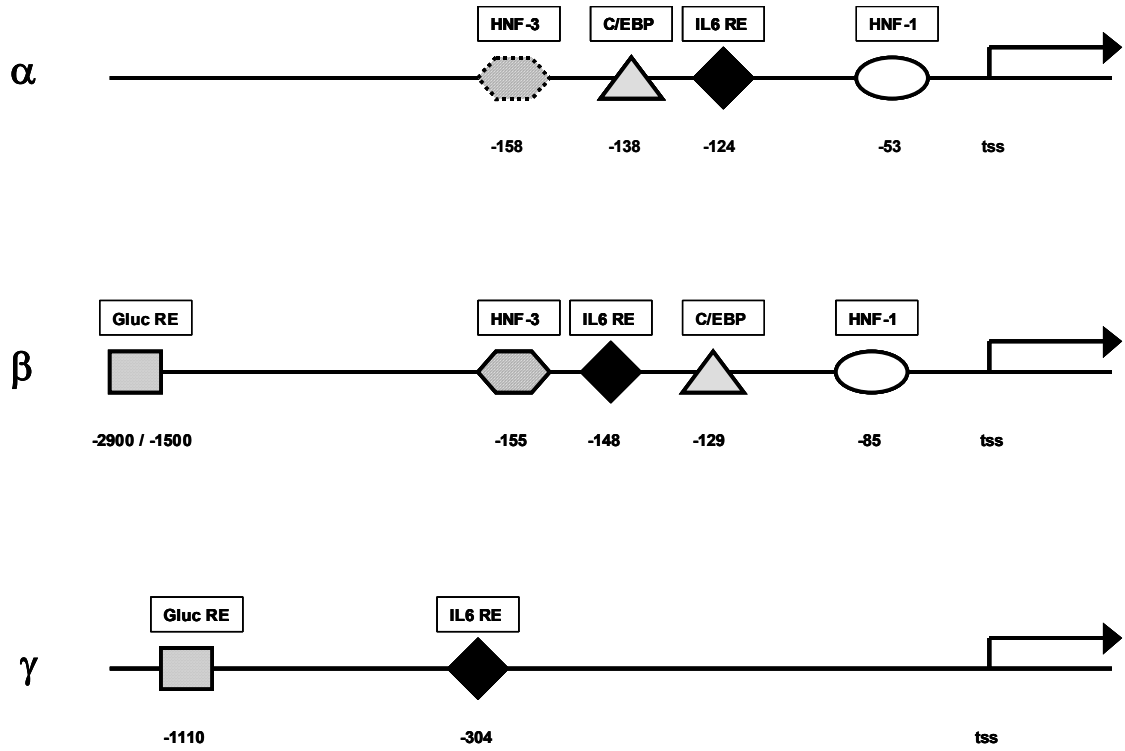

Figure 2: Schematic representation of the regulatory elements in the human fibrinogen α, β and γ chain gene promoters. Locations relative to the transcription start site are shown. Identification of the HNF-3 site in the fibrinogen β promoter is described in chapter 2 of this thesis, and the HNF-3 site depicted in the fibrinogen α promoter is a putative HNF-3 site.

GENETIC VARIATION OF THE FIBRINOGEN β GENE AND PLASMA LEVELS OF FIBRINOGEN

Fibrinogen plasma levels are partly genetically determined, and a recent study of twins estimated the heritability of fibrinogen plasma levels at 45% [45]. Several genetic variations have been described in all three fibrinogen genes [46,47]. The -455G/A polymorphism in the fibrinogen β promoter has been studied most often, and the -455A allele has been associated with elevated habitual plasma fibrinogen levels in many studies [48-54]. Because elevated plasma levels of fibrinogen are associated with an increased risk of arterial cardiovascular disease, the relationship between polymorphisms in the fibrinogen gene and arterial disease has also been investigated. Although the association between fibrinogen plasma levels and genetic variation is fairly consistent, the relationship between polymorphisms in the fibrinogen β gene and arterial disease turns out to be much weaker (meta-analysis by Boekholdt *et al* [55] and reviewed by Folsom [21]). However, there is evidence that the effect of genetic variation on plasma fibrinogen levels may be much more relevant under specific circumstances, and the presence of an interaction between genetic variation in the fibrinogen β promoter and inflammation has been suggested. Some studies have investigated the association between fibrinogen β genotype

and the response of fibrinogen levels to inflammatory triggers. These studies consistently report a stronger rise in fibrinogen levels in -455A allele carriers than in GG homozygotes, after exposure to strong acute phase stimuli such as trauma, strenuous exercise and surgery. However, it should be noted that the sample size of most of these studies has been small [27-29,56].

Change from G to A at position -455 in the fibrinogen β promoter abolishes a recognition site for the restriction enzyme *Hae*III site, and therefore determination of this polymorphism is simple. In the first studies on the relationship between fibrinogen plasma levels and genetic variation, positive results were obtained for the easily detectable -455G/A variation, and therefore this polymorphism has been investigated in the vast majority of the epidemiological surveys up until now. However, the -455G/A variation is in complete linkage disequilibrium with several other polymorphisms in Caucasians, including the -1420G/A, -993C/T, and -148C/T variations in the promoter region of the fibrinogen β gene (Figure 3) [57]. Due to this allelic association, any of these other polymorphisms (or even a linked polymorphism located outside the promoter region of the fibrinogen β gene) could be responsible for the effects observed to be associated with the -455G/A alleles.

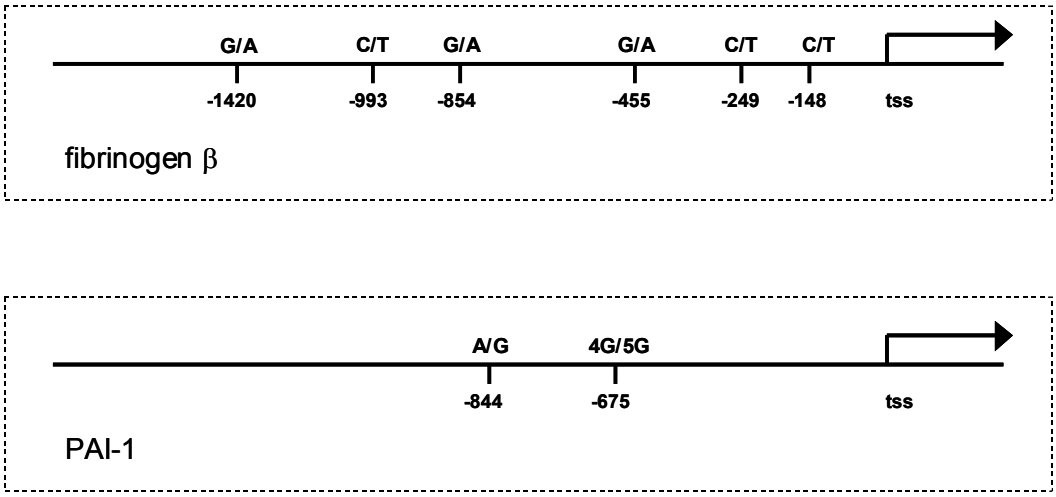

Figure 3: Schematic representation of the promoter regions of the fibrinogen β chain and PAI-1 genes. The locations of the common polymorphisms are indicated.

PLASMINOGEN ACTIVATOR INHIBITOR-1

THE PLASMINOGEN ACTIVATOR INHIBITOR-1 PROTEIN AND GENE

Plasminogen activator inhibitor-1 (PAI-1) is a glycoprotein with a molecular weight of 50 kD and it is a member of the serine protease inhibitor (serpin) family. The PAI-1 gene is located on chromosome 7. Many cell types, including

hepatocytes, endothelial cells, fibroblasts, and smooth muscle cells produce PAI-1, but the relative importance of the contribution of these cell types to PAI-1 plasma levels *in vivo* is still unclear. Expression of PAI-1 is mediated by several factors such as hormones [58,59], the cytokines TNF-α, IL-1 and TGF-β [60-62], and several other factors including very low density lipoprotein (VLDL), fatty acids and glucose [63-65]. PAI-1 exists in two forms in plasma, and the active form is stabilized by binding to vitronectin. PAI-1 controls fibrinolysis by a rapid inhibition of tissue-type plasminogen activator (t-PA), which is the major physiological activator of fibrinolysis, and increased PAI-1 levels result in impaired fibrinolytic capacity [66]. Elevated PAI-1 levels have been associated with cardiovascular disease in several epidemiological studies [21,67,68]. Elevated PAI-1 levels reflect a decreased fibrinolytic state and there is also evidence that PAI-1 can contribute causally to the development of cardiovascular disease. In transgenic mouse studies, wild-type mice developed thrombosis over two times faster than mice deficient in PAI-1, after injury to the carotid arteries [69]. Elevated PAI-1 levels are associated with the features of the metabolic syndrome (e.g. elevated BMI, hypertension, hyperinsulinaemia, lipid disorders and increased cytokine levels, reviewed by Juhan-Vague and Alessi [70]). This indicates that elevated PAI-1 levels, cardiovascular disease and several traditional cardiovascular risk factors are strongly related and may even represent different aspects of the same phenomenon.

GENETIC VARIATION OF THE PAI-1 GENE AND PLASMA LEVELS OF PAI-1

The heritability of PAI-1 levels has been estimated at 50-60% [21,71]. Similarly as described above for the fibrinogen β gene, also in the PAI-1 gene several common genetic polymorphisms have been identified that are associated with the plasma levels of the protein [72]. Because of this association and the fact that elevated plasma levels of PAI-1 are associated with an increased risk of arterial disease, the relationship also between the PAI-1 polymorphisms and arterial cardiovascular disease has been investigated. The -675(4G/5G) polymorphism in the promoter region of the PAI-1 gene has been investigated on many occasions. The -675(4G) allele has been associated with elevated plasma PAI-1 levels, and occasionally also with an increased risk of cardiovascular disease [73-77]. As is also the case for fibrinogen, the relationship between polymorphisms and plasma protein levels is fairly consistent, but the relationship between polymorphisms and arterial disease is again much weaker (meta-analysis by Boekholdt *et al* [55] and reviewed by Folsom [21]).

For PAI-1 also there is evidence that genetic variation in the promoter region of the gene may modulate the response of the gene to environmental triggers. There are indications that PAI-1 promoter genotype can influence the relationship between PAI-1 plasma levels and metabolic factors such as levels of triglycerides, insulin and plasma lipoproteins [72,78,79], but there are also studies that could not detect any such effect of the PAI-1 promoter genotype [80,81].

Another common polymorphisms in the promoter region of the PAI-1 gene is the –844A/G variation, which is also located in the promoter region and is in strong, but not complete linkage disequilibrium with the -675(4G/5G) polymorphism (Figure 3) [82].

THIS THESIS

Evidence is emerging that genetic variation can not only change the constitutive protein expression, but may also influence the response of genes to external triggers, thereby possibly influencing the susceptibility of an individual to cardiovascular disease.

In addition, it has been shown repeatedly that elevated fibrinogen plasma levels, and PAI-1 plasma levels are risk factors for cardiovascular disease.

> *The aim of this thesis was to identify functional polymorphisms in the fibrinogen β and PAI-1 promoters, to determine their interaction with environmental factors, and to elucidate the underlying mechanisms.*

This thesis includes results of population-based association studies on the relationship between genetic variation and plasma protein levels, and the results of *in vitro* functional assays explaining the molecular mechanisms.

In **chapter 2** functional *in vitro* assays investigating the regulation of fibrinogen β promoter activity are described. A new binding site for the transcription factor HNF-3 is identified and its importance for the regulation of fibrinogen β promoter activity by the pro-inflammatory cytokine IL6 is illustrated. The activity of this HNF-3 binding site is influenced by genetic variation, which is explained in more detail in the next chapter. In **chapter 3**, the effect of fibrinogen β promoter haplotype on IL6-induced and basal fibrinogen β transcriptional activity is demonstrated using *in vitro* functional assays, and the -148C/T polymorphism is identified as the functional variation. The effect of the -148C/T variation on the response of the fibrinogen β promoter to IL6 is explained by the effect of this polymorphism on the activity of the newly identified HNF-3 element. **Chapter 4** describes the results of a small *in vivo* association study. The interindividual variation in response by fibrinogen-, C- reactive protein- and IL6 plasma levels to a standardized inflammatory trigger is quantified in healthy individuals, and the contribution of genetic variation in the IL6 and fibrinogen β promoters to this interindividual variation is explored. **Chapter 5** describes population-based association studies and *in vitro* functional assays, both on the effect of genetic variation in the PAI-1 promoter on PAI-1 expression levels. In the association studies we observed a significant relationship between PAI-1 promoter haplotype and PAI-1 antigen levels in lean individuals. However, no direct effect of PAI-1 promoter haplotype on PAI-1 promoter activity was detected *in*

vitro. **Chapter 6** includes a description of the associations of several environmental and genetic factors with PAI-1 plasma levels, in a population of healthy Dutch individuals. In **chapter 7** the results described in this thesis are discussed and related to insights from the literature.

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Chapter 2

Hepatocyte nuclear factor-3 (HNF-3) is important for interleukin-6-induced fibrinogen β expression; evidence for a novel role of HNF-3 transcription factors

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Submitted (combined with chapter 3)

Hepatocyte nuclear factor-3 (HNF-3) is important for interleukin-6-induced fibrinogen β expression; evidence for a novel role of HNF-3 transcription factors

Maartje Verschuur, Maureen de Jong, Moniek P.M. de Maat and Hans L. Vos

ABSTRACT

Inflammation is an important process in the development of cardiovascular disease and therefore understanding the inflammatory response of cardiovascular risk factors is essential. Elevated plasma fibrinogen levels are an independent risk factor for cardiovascular disease, and the most important inducer of fibrinogen expression is the inflammatory mediator interleukin-6 (IL6). The aim of the present study was to gain more understanding of the hepatic acute phase response of fibrinogen. Using reporter gene assays and mutation analysis, we identified a functional HNF-3 binding motif located at –159/-151 in the fibrinogen β promoter. This HNF-3 site is essential for the full response of the gene to IL6, but is not required for basal promoter activity. EMSA experiments with HepG2 nuclear extracts revealed that HNF-3β binds to the HNF-3 site. Furthermore we show with functional assays that the activity of the IL6-responsive C/EBP site, located at -133/-125, is dependent on the integrity of the adjacent HNF-3 site and *vice versa*. The dependency of C/EBPβ on the functional HNF-3 site can explain the necessity of this HNF-3 site in the IL6 response. In this study we show the involvement of HNF-3 in cytokine-induced gene response, which is a new function for this family of transcription factors.

INTRODUCTION

Inflammation is an important process in the development of cardiovascular disease, and increased plasma levels of several acute phase proteins including fibrinogen are consistently associated with an increased risk of cardiovascular disease [1-5]. Inflammatory factors play a dual role. On the one hand their plasma levels reflect the severity of inflammatory processes in the vessel wall, and on the other hand inflammatory factors can contribute directly to the development of disease. Fibrinogen is found in the atherosclerotic plaque where it can contribute to the progression of atherosclerosis, for example by increasing the chemotaxis of smooth muscle cells [6] and affecting the stability and structure of the plaque [7-9]. Because of the relationship of fibrinogen to atherosclerosis and cardiovascular events, much attention has been paid to the regulation of fibrinogen levels, both under basal and inflammatory conditions.

The mature fibrinogen molecule is composed of 3 pairs of polypeptide chains: two α chains, two β chains and two γ chains, and *in vitro* functional studies showed that synthesis of the β chain is rate-limiting [10]. Fibrinogen is expressed by the liver. Fibrinogen levels can strongly increase in response to intense acute phase stimuli such as trauma, surgery, or strenuous exercise, and fibrinogen levels are chronically elevated in the presence of mild (inflammatory) stimuli such as smoking or severe atherosclerosis [11-13]. Interleukin-6 (IL6) is the main mediator of acute phase-induced fibrinogen synthesis, and sequences responsive to IL6 are present in the promoter regions of the genes coding for the three fibrinogen chains. In the promoter region of the fibrinogen β gene, several DNA sequences that are required for full IL6-induced expression have been identified; an hepatocyte nuclear factor-1 (HNF-1) site at approximately 85 nucleotides upstream of the transcription start site, a CCAAT box/ enhancer-binding protein- (C/EBP) binding site, and an IL6 responsive element (IL6 RE) [14-16]. The C/EBP binding site and the IL6 RE are located adjacent to each other at approximately 125 nucleotides upstream from the transcription start site. C/EBPβ (also named LAP or NF-IL6) has been identified as a transcription factor important in mediating the IL6 response of many liver acute phase genes (reviewed by Poli [17]) and the binding of C/EBPβ to the fibrinogen β promoter has been demonstrated [15]. The IL6 RE is located 4 base pairs upstream of the C/EBPβ site, and it has been shown repeatedly that sequence changes in this element result in the loss of response of the gene to IL6 [14-16]. However, no transcription factor binding to this IL6 RE has been identified yet leaving the regulation of the hepatic IL6-induced fibrinogen β expression partly obscure.

We here report the identification of a novel hepatocyte nuclear factor-3 (HNF-3) binding site in the fibrinogen β promoter. This HNF-3 site is located just upstream of the IL6 RE and the C/EBP binding site, and it is directly involved in the hepatic IL6 response of the fibrinogen β gene. The newly identified HNF-

3 site and the previously described C/EBP site mutually affect each other and this can explain the importance of this HNF-3 site for IL6-induced fibrinogen β expression. A direct involvement of HNF-3 (also named FoxA) in cytokine-induced gene response has not been described before.

METHODS

Materials

CELL LINES

The human hepatoma cell lines HepG2 (American Type Culture Collection) and HuH7 [18] were maintained in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Bio Whittaker Europe) and 10% foetal bovine serum (FBS, Invitrogen).

PGL3-FIBRINOGEN β PROMOTER REPORTER GENE CONSTRUCTS

1800 bp of the promoter region of the fibrinogen β gene (-1788 to +8, taking position 1500 in Genbank accession number X05018 as +1, the transcription start site) was amplified by PCR on genomic DNA. In this PCR reaction 200 µM of each dNTP, 10 pmol of each primer (forward primer: 5'-TCT TAC GCG TGA AGA ATG CCA ATC AGA GTA-3', reverse primer: 5'-TCA TCT CGA GTA GAC TTA ACT GAG AGA TCT TCA-3'), 3.5 U of High Fidelity polymerase (Roche) and 50 ng of genomic DNA in a total volume of 50 µl was used. The PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 4 min, and a final extension at 72°C for 5 min. PCR products were digested with *Mlu*I and *Xho*I (the restriction sites introduced are underlined in the primer sequences) and cloned into the *Mlu*I and *Xho*I sites of pGL3- basic (Promega), resulting in the pGL3-FGB wild-type construct. The putative HNF3 site at –159/-151 in the fibrinogen β promoter was mutated by site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene) as described by the manufacturer, using 10-30 ng of pGL3-FGB wild-type construct as template and the mutagenic primers with sequences 5'-GCA ACA TCT TCC CAG CAA AGC TGA AGT ACT TGT CAT ACA AC -3' and 5'-GTT GTA TGA CAA GTA CTT CAG CTT TGC TGG GAA GAT GTT GC –3'. This resulted in a pGL3-basic-derived fibrinogen β promoter construct with the core of the putative HNF3 site at –159/-151 changed from TATTTACTT to GAAGTACTT (pGL3-FGB HNF3 mut). The identity of the clones was verified by sequencing. In addition to the constructs described above, constructs designed to study the C/EBP site at -133/-125 were also used. These constructs were already available at our institute and are described elsewhere [16]. These pGL3-basic-derived constructs include 400 bp of the wild-type fibrinogen β promoter,

or a 400 bp fibrinogen β promoter fragment with the core C/EBP site at –133/-125 changed from GTTGCTTAA to GTTTAGTAA (pGL3-FGB C/EBP mut). The sequences of the region spanning from -165 to -115 of the newly created constructs (including 1800 bp promoter fragments), and the available constructs (including 400 bp promoter fragments), are shown in Table 1.

VECTORS EXPRESSING TRANSCRIPTION FACTORS

Vectors expressing HNF-3α, HNF-3β, HNF-3γ and C/EBPβ (*pCDNA3.1-HNF3α, pCDNA3.1-HNF3β, pCDNA3.1-HNF3γ, pSCT-C/EBPβ*, all from rat origin) and their empty counterparts (*pCDNA3.1-, pSCT-*) were a kind gift from Dr. P. Holthuizen (Department of Physiological Chemistry, University of Utrecht, the Netherlands).

Table 1: Wild-type and mutant fibrinogen β promoter sequences

Fibrinogen β promoter sequence	
	HNF-3 *IL6 RE* *C/EBP* 5′-GTATGACAAGTAAATAAGCTTTGCTGGGAAGATGTTGCTTAAATGATA-3′ 3′-CATACTGTTCATTTATTCGAAACGACCCTTCTACAACGAATTTACTAT-5′
Consensus sequence [15,20]	3′-YTYRKTTAT-5′ 5′-CTTGCNNAA-3′
Mutations created in fibrinogen β pGL3-basic constructs	
pGL3-FGB HNF3 mut (1800 bp promoter fragment)	5′-GTATGACAAGTA**CT**T**C**AGCTTTGCTGGGAAGATGTTGCTTAAATGATA-3′ 3′-CATACTGTTCAT**GA**A**G**TCGAAACGACCCTTCTACAACGAATTTACTAT-5′
pGL3-FGB CEBP mut (400 bp promoter fragment)	5′-GTATGACAAGTACTTCAGCTTTGCTGGGAAGATGTT**TAG**TAAATGATA-3′ 3′-CATACTGTTCATGAAGTCGAAACGACCCTTCTACAA**ATC**ATTTACTAT-5′
Sequences of oligonucleotides used in EMSAs	
Fib β wild-type oligo	5′-TATGACAAGTAAATAAGCTTTGCTGG-3′ 3′-ATACTGTTCATTTATTCGAAACGACC-5′
Fib β HNF3 mut oligo	5′-TATGACAAGTA**CT**T**C**AGCTTTGCTGG-3′ 3′-ATACTGTTCAT**GA**A**G**TCGAAACGACC-5′
HNF3 consensus oligo	5′-GCCCATTGTTTGTTTTAAGCC-3′ 3′-CGGGTAACAAACAAAATTCGG-5′
Consensus sequence [31]	5′-VAWTRTTKRYTY-3′

The sequence of the wild-type fibrinogen β promoter, the consensus sequences of HNF-3 and C/EBP binding sites, the mutations in the reporter gene constructs, and the complete sequences of the oligonucleotides used in EMSA experiments are shown. Note that different HNF-3 consensus sequences have been described, the one present in the fibrinogen β promoter is different from the one represented by the HNF-3 consensus oligonucleotide [20,31].

ANTIBODIES

Polyclonal antibodies directed against HNF-3β (M-20 sc-6554X) and HNF-3γ (N-19 sc-5361X) were obtained from Santa Cruz Biotechnology, and antibodies directed against HNF-3α (2000007) were obtained from Geneka.

Luciferase-reporter gene assays

TRANSFECTION CONDITIONS

HepG2 and HuH7 cells were plated in 24-wells plates in DMEM with 10% FBS at a density of 1.0 *10^5 cells per well. After allowing the cells to attach overnight, the medium was replaced with serum-free medium supplemented with 0.1% human serum albumin (HSA, Cealb®). After 2 h, cells were transfected using FuGene 6 (Roche), according to the manufacturer's protocol. 200 ng of pGL3 construct, 4 ng of pRL-tk (Renilla luciferase expression construct, Promega) were used per well. If applicable, vector expressing HNF-3α, HNF-3β, HNF-3γ, C/EBPβ, or a molar equivalent of empty expression vector as control were added. The total amount of DNA was kept at 400 ng per well with carrier DNA (herring sperm, Invitrogen). The effect of the empty expression vectors on fibrinogen β promoter activity and on the Renilla luciferase expression was determined for all conditions tested in this study, and no effects of the empty control vectors were detected. For each construct at least two independent DNA preparations were used, and all DNA preparations were transfected at least twice in triplicate. 24 hrs after transfection the medium was replaced with DMEM + 0.1% HSA, containing IL6 concentrations ranging from 0 to 2.5 ng/ml (recombinant human IL6, Pepro Tech).

LUCIFERASE ASSAY

After culturing the cells for 24 hrs in the presence of IL6, cells were washed with 500 µl phosphate-buffered saline (PBS) and subsequently lysed for 15 minutes on a rotary platform at room temperature with 100 µl Passive Lysis Buffer (PLB, Promega). The Firefly luciferase reporter and Renilla luciferase internal control activities were measured in 10 µl lysate, using the Dual-Luciferase® Reporter Assay System (Promega). Luminescence was measured using a luminometer (Berthold).

Electrophoretic Mobility Shift Assay (EMSA)

PREPARATION OF NUCLEAR EXTRACTS

HepG2 cells were cultured under serum-free conditions (DMEM P/S + 0.1% HSA) for 24h prior to preparation of the nuclear extracts. For the preparation of nuclear extracts of IL6-stimulated cells, cells were incubated with 5 ng/ml IL6 for 15 minutes. Cells were washed and lysed, and nuclear extracts were prepared according to the method of Slomiany *et al* [19]. Buffers were

supplemented with protease inhibitor cocktail (Complete™ Mini, Roche), and with phosphatase inhibitors (Na-orthovanadate, final concentration 250 μM; β-glycerophosphate, final concentration 25 mM). The protein concentration in the nuclear extracts was estimated using the BCA micro kit (Pierce), and the samples were stored at –80°C for future use.

OLIGONUCLEOTIDES

Double stranded 26 bp oligonucleotides including the wild-type HNF-3 site (Fib β wild-type) or the mutated HNF-3 site (Fib β HNF3 mut) were designed. In addition, a double stranded 21 bp HNF-3 consensus oligonucleotide was designed according to the consensus sequence provided by Locker *et al* [20] (Table 1). Pairs of complementary oligonucleotides were annealed at equimolar amounts and radioactively labelled at the 5' ends with γ-^{32}ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Invitrogen). The double-stranded and labelled oligonucleotides were purified using MicroSpin G-25 Columns (Amersham Pharmacia Biotech), and stored at –20°C for future use.

BINDING REACTION AND ELECTROPHORESIS

For each binding reaction, 3 μg of nuclear extract was pre-incubated for 30 minutes on ice with 5 μg poly dIdC (Amersham Pharmacia Biotech) in a 12 μl reaction mixture containing 10 mM Hepes (pH 7.9), 100 mM KCl, 25 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM EDTA, 0.1% (v/v) NP40, 10% (v/v) glycerol, 0.03 mg/ml BSA, 250 μM Na-orthovanadate and 25 mM β-glycerophosphate. Subsequently, the labelled oligonucleodides were added to the pre-binding reactions and this mixture was incubated for another 30 minutes on ice. After this and if applicable, 3 μg of antibody directed against the HNF-3 isoforms was added to the binding reaction and incubated for an additional 30 minutes on ice. Competition assays were performed by adding a 100-fold molar excess of unlabelled double-stranded oligonucleotides to the radioactive oligonucleotides, prior to their addition to the binding reaction. The reaction products were loaded onto 5% non-denaturing polyacrylamide gels and run in 0.25x TBE (1x TBE = 0.1M Tris, 0.09M Boric Acid, 0.001M EDTA). Gels were blotted on Whatmann paper and bands were visualized by autoradiography.

STATISTICAL METHODS

Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity as the internal standard. These normalized luciferase activity levels were expressed as a percentage of the normalized luciferase activity of the pGL3-FGB wild-type construct at baseline. Normalized expression levels of the wild-type fibrinogen β promoter constructs were compared with the normalized expression levels of the mutant constructs using Student's *t*-test; SPSS 11.0 for Windows was used. P-values are described in the figure legends.

RESULTS

A nearly perfect HNF-3 consensus sequence was identified after a search for putative transcription factor binding sites with MatInspector Professional [21]. This putative HNF-3 site is located at position -159/-151 in relation to the transcription start site in the fibrinogen β promoter, just upstream of the C/EBP binding site and the IL6 RE (Table 1). This region of the fibrinogen β promoter is highly conserved during mammalian evolution, as an alignment of human, mouse and rat sequences indicated (data not shown). Because of our interest in the possible role of this site in the regulation of the IL6 response of fibrinogen, the functionality of this putative HNF-3 site was investigated.

All HNF-3 family members transactivate the fibrinogen β promoter

The pGL3-FGB wild-type construct including 1800 bp of the proximal fibrinogen β promoter was transfected into HepG2 cells in the presence of one of the overexpressed HNF-3 isoforms, and the luciferase activity was determined. The fibrinogen β promoter responded strongly to all HNF-3 isoforms. Promoter activity increased 2-fold in the presence of HNF-3α, 9-fold in the presence of HNF-3β and up to 5-fold in the presence of HNF-3γ, when a maximum of 500 pg of vector expressing the HNF-3 isoforms was cotransfected (Figure 1). This demonstrates that the fibrinogen β promoter is activated by overexpression of HNF-3 isoforms, which may be explained by the presence of a functional HNF-3 site in the first 1800 bp of the fibrinogen β promoter.

Identification of an HNF-3 responsive site at –159/-151 in the fibrinogen β promoter

To explore whether the putative HNF-3 site at position –159/ -151 is functional, the core sequence of this site was changed from TATTTACTT to GAAGTACTT by site-directed mutagenesis, and the mutant luciferase constructs were transfected into HepG2 cells. No important effect of mutation of the HNF-3 site on basal expression was observed (Figures 1, 3a and 4a). Cotransfection experiments with vectors expressing the HNF-3 isoforms revealed that mutation of the putative HNF-3 site resulted in a significant loss of response of the promoter to overexpressed HNF-3. After mutation, the fibrinogen β promoter did not respond to HNF-3α any more, the maximal response to HNF-3β had decreased from 9-fold to 3-fold, and the maximal response to HNF-3γ had decreased from 5-fold to 3-fold (Figure 1). These results from experiments with mutant constructs show that the HNF-3 site at –159/-151 in the fibrinogen β promoter is functional, but apparently not essential for basal promoter activity. Similar results were obtained in another liver-derived cell line, HuH7. However, the response of the fibrinogen β promoter to overexpressed HNF-3 family members was weaker in HuH7 cells than in HepG2 cells (data not shown).

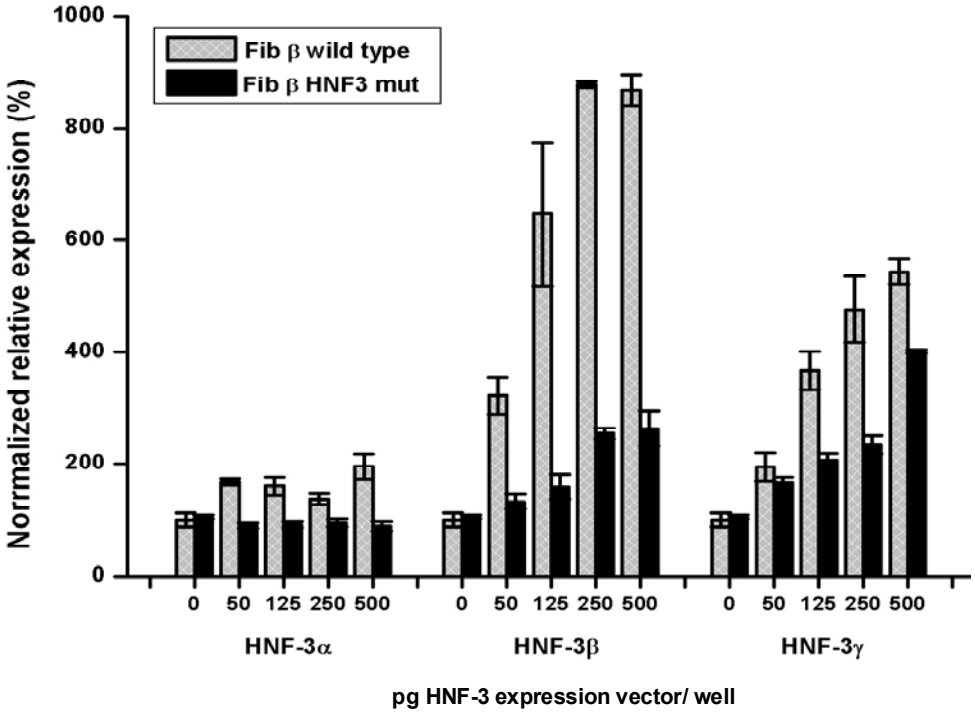
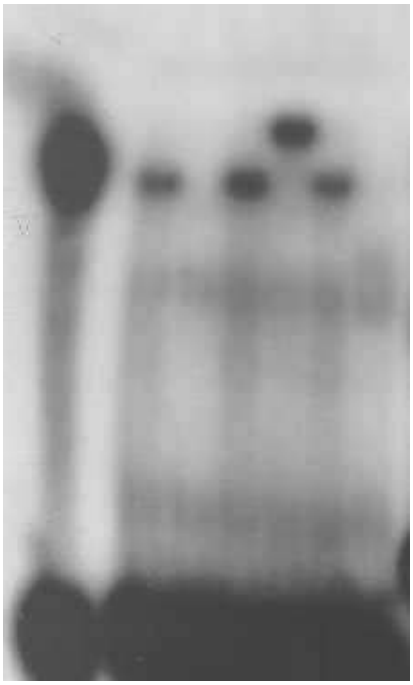


Figure 1: Transactivation of wild-type and mutant fibrinogen β promoter variants by HNF-3 isoforms and identification of a functional HNF-3 site at -159/-151. Fibrinogen β promoter reporter gene constructs were transfected into HepG2 cells, in the presence of increasing amounts of HNF-3α, HNF-3β or HNF-3γ expression vector. Differences in activity between the wild-type and HNF-3 mutant were significant at all HNF-3α and at all HNF-3β concentrations, and at concentrations above 50 pg of HNF-3γ expression vector ($p<0.005$ in all these cases). Normalized luciferase activities are expressed in relation to baseline activity of the wild-type promoter construct and means (± SDs) of triplicate transfections are shown.

HNF-3β binds to the –159/-151 sequence in the fibrinogen β promoter

To detect nuclear proteins binding to the HNF-3 responsive site in the fibrinogen β promoter, electrophoretic mobility shift assays were performed (Figure 2). When oligonucleotides representing the wild-type HNF-3 element (Fib β wild-type), the mutant HNF-3 element (Fib β HNF3 mut) or an HNF-3 consensus site were incubated with HepG2 nuclear extracts, binding of a nuclear complex to the HNF-3 consensus oligonucleotide (lane 1) and to the fibrinogen β wild-type oligonucleotide (lane 3) was observed. However, no binding of nuclear proteins to the fibrinogen β mutant oligonucleotide was observed (lane 8). The complex on the fibrinogen β wild-type oligonucleotide disappeared when 100-fold excess of cold HNF-3 consensus oligonucleotide was added, showing that the nuclear protein binding the –159/-151 sequence in the fibrinogen β promoter also recognizes this HNF-3 consensus sequence (lane 4). The mobility of the complex was retarded when an antibody directed against HNF-3β was added (lane 6), but not when antibodies directing against HNF-3α

(lane 5) or HNF-3γ (lane 7), were added. These results show that in HepG2 nuclear extracts, the protein binding to the HNF-3 responsive site at –159/-151 in the fibrinogen β promoter is HNF-3β.



	1	2	3	4	5	6	7	8
Cold HNF-3 cons	-	-	-	+	-	-	-	-
Anti-HNF-3α	-	-	-	-	+	-	-	-
Anti-HNF-3β	-	-	-	-	-	+	-	-
Anti-HNF-3γ	-	-	-	-	-	-	+	-

Figure 2: Binding of HNF-3β to the HNF-3 site at –159/-151 in the fibrinogen β promoter. Labelled oligonucleotides representing the HNF-3 site in the wild-type fibrinogen β promoter (lane 3, 4, 5, 6, 7), the mutated HNF-3 site in the fibrinogen β promoter (lane 8), or the HNF-3 consensus oligonucleotide (lane 1) were incubated with nuclear extracts derived from HepG2 cells cultured under basal conditions. 100-fold molar excess of cold HNF-3 consensus oligonucleotide was added in lane 4, and antibodies against HNF-3α (lane 5), HNF-3β (lane 6) and HNF-3γ (lane 7) were used, and lane 2 was left empty. The locations of HNF-3 and the supershifted complexes (ss) are indicated by arrows.

Integrity of the HNF-3 site is important for IL6-induced fibrinogen β promoter activity

The HNF-3 site identified at –159/-151 is located next to previously described IL6 responsive sequences, and therefore the function of the HNF-3 site in IL6-induced fibrinogen β promoter activity was studied. The addition of 2.5 ng/ml IL6 to HepG2cells transfected with reporter gene constructs containing 1800

bp of the proximal wild-type fibrinogen β promoter resulted in an increase of 12-fold of basal promoter activity (Figure 3a). The promoter variant with the mutated HNF-3 site however, responded to this IL6 concentration with a smaller increase of 5-fold only. Also at lower IL6 concentrations, mutation of the HNF-3 site reduced the IL6 response of the fibrinogen β promoter approximately by a factor of 2.5. These results show that this HNF-3 site is important for IL6-induced fibrinogen β promoter activity. Similar results were obtained in HuH7 cells, although the overall response to IL6 of the fibrinogen β promoter constructs was again weaker in HuH7 cells than in HepG2 cells (data not shown).

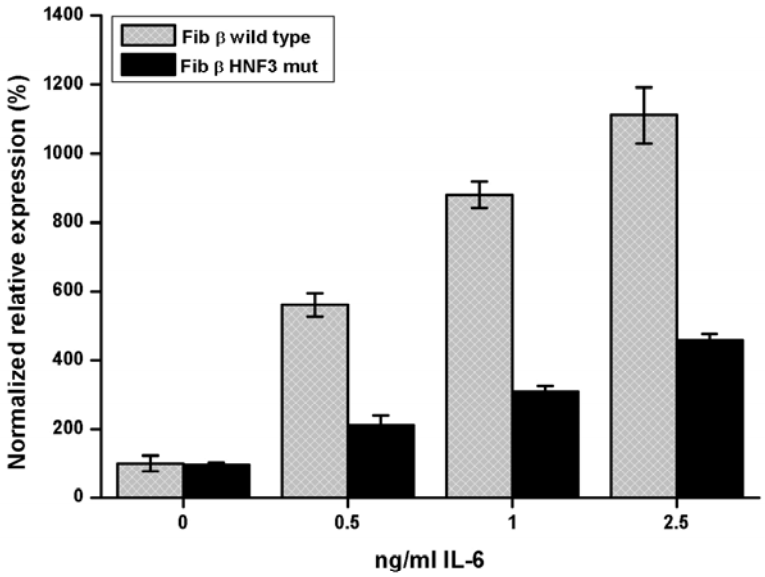
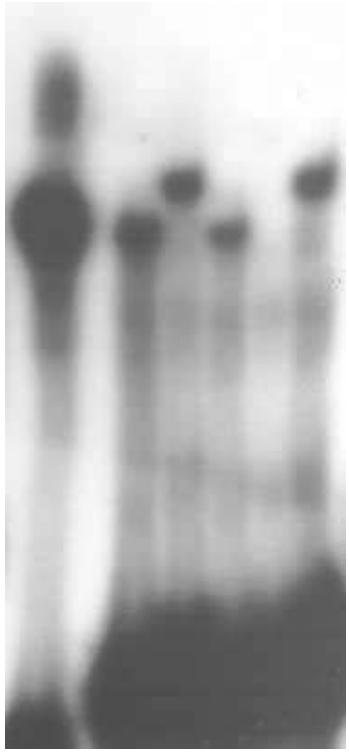


Figure 3a: The HNF-3 site at −159/-151 in the fibrinogen β promoter is required for the full response of the fibrinogen β promoter to IL6. Fibrinogen β promoter reporter gene constructs were transfected into HepG2 cells, and cells were subsequently treated with IL6. At all IL6 concentrations, the IL6-induced activity of the wild-type promoter was significantly higher than the induced activity of the HNF-3 mutant promoter ($p<0.0003$ in all these cases). Normalized luciferase activities are expressed in relation to baseline activity of the wild-type promoter construct, and means (± SDs) of triplicate transfections are shown.

Binding of HNF-3β under IL6-induced conditions

To investigate whether IL6 influences the binding of HNF-3β to the fibrinogen β promoter, electrophoretic mobility shift assays were performed with basal (lanes 1, 3 and 4) and IL6-treated (lanes 5 to 7) HepG2 nuclear extracts (Figure 3b). The binding of a single complex to the fibrinogen β wild-type oligonucleotide (Fib β wild-type) was observed under basal (lane 3) and IL6-induced conditions (lane 5).



	1	2	3	4	5	6	7
Basal NE	+	-	+	+	-	-	-
+IL6 NE	-	-	-	-	+	+	+
Cold HNF-3 cons	-	-	-	-	-	+	-
Anti-HNF-3β	-	-	-	+	-	-	+

Figure 3b: Binding of HNF-3β to the HNF-3 site at –159/-151 in the fibrinogen β promoter under IL6-induced conditions. Labelled oligonucleotides representing the HNF-3 site in the wild-type fibrinogen β promoter (lane 3, 4, 5, 6, 7) or the HNF-3 consensus oligonucleotide (lane 1) were incubated with nuclear extracts (NE) derived from HepG2 cells cultured under basal conditions (lane 1, 3, 4) or in the presence of IL6 (lane 5, 6, 7). 100-fold molar excess of cold HNF-3 consensus oligonucleotide was added in lane 6, and antibodies against HNF-3β were used in lane 4 and 7. Lane 2 was left empty. The locations of HNF-3 and the supershifted complexes (ss) are indicated by arrows.

The complex binding under IL6-induced conditions disappeared when 100-fold excess of cold HNF-3 consensus oligonucleotide was added (lane 6) and was retarded after the addition of an antibody directed against HNF-3β (lane 7), identifying this nuclear complex as HNF-3β. These results were similar to what was observed under basal conditions, so there was no qualitative effect of IL6 treatment on the binding of HNF-3β to the HNF-3 element in the fibrinogen β promoter.

Interaction between the HNF-3 and C/EBP sites
To investigate whether the HNF-3 site and the C/EBPβ site interact, transfection experiments were performed with both the wild-type and the mutated fibrinogen β promoter constructs in the presence of overexpressed C/EBPβ. The wild-type fibrinogen β promoter responded strongly to overexpressed C/EBPβ, but mutation of the HNF-3 site severely reduced the responsiveness of the fibrinogen β promoter to C/EBPβ (Figure 4a). When 250 pg of vectors expressing C/EBPβ were cotransfected, the presence of the mutated HNF-3 site decreased the maximal response from 7-fold to 3-fold. A similar effect of the mutation was also observed at lower C/EBPβ concentrations.

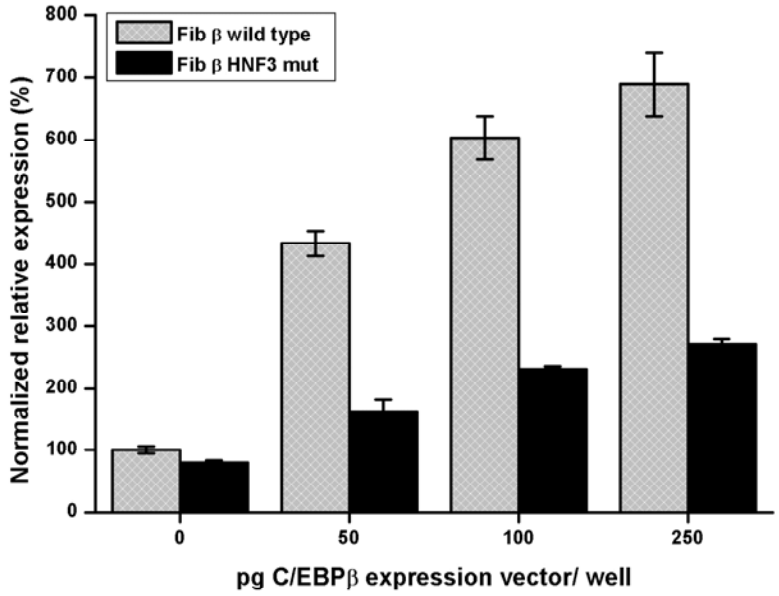


Figure 4a: The HNF-3 site and the C/EBP site in the fibrinogen β promoter interact with each other. Fibrinogen β promoter reporter gene constructs were transfected into HepG2 cells. Constructs including 1800 bp of the fibrinogen β promoter (wild-type or HNF3 mutant) were transfected together with increasing amounts of C/EBPβ expression vector. At all C/EBPβ expression vector concentrations, the C/EBPβ-induced activity of the wild-type promoter was significantly higher than the induced activity of the HNF-3 mutant promoter ($p<0.0003$ in all these cases)

This shows that the response of the fibrinogen β promoter to C/EBPβ is dependent on the integrity of the HNF-3 site at –159/-151. However, mutation of the HNF-3 site strongly decreases the response to C/EBPβ but does not completely abolish the response. This indicates that the HNF-3 site is very important for the C/EBP response, but is not an absolute requirement.

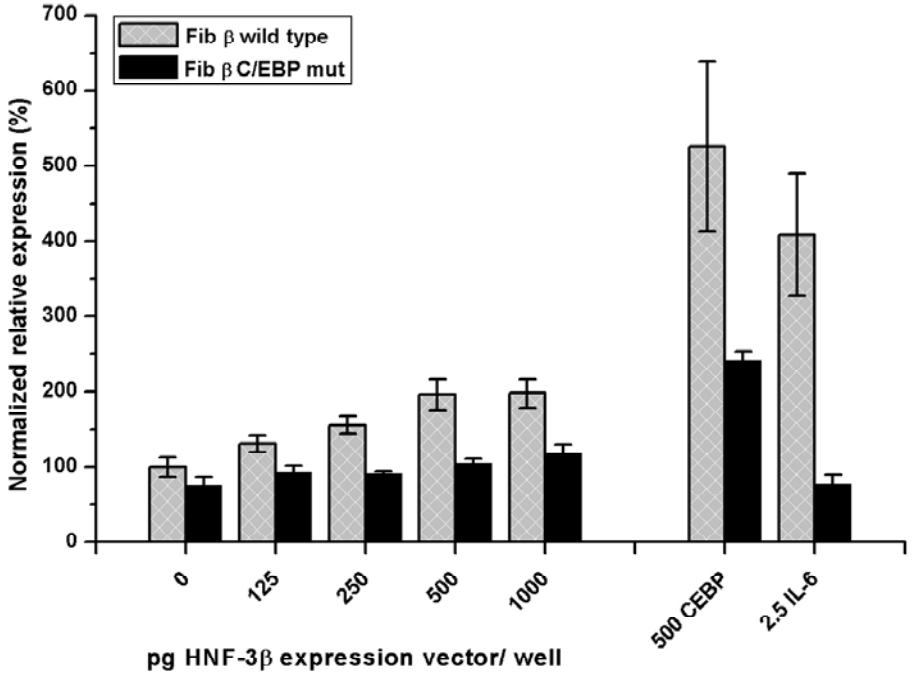


Figure 4b: The C/EBP site and the HNF-3 site in the fibrinogen β promoter interact with each other. Constructs including 400 bp of the fibrinogen β promoter (wild-type or C/EBP mutant) were transfected together with increasing amounts of HNF-3β expression vector, and the differences in activity between the wild-type promoter and the C/EBP mutant were significant at all HNF-3β expression vector concentrations ($p<0.005$). (left panel). Constructs including the wild-type or C/EBP mutant fibrinogen β promoter (400 bp fragments) were transfected in the presence of IL6 or overexpressed C/EBPβ. The wild-type promoter responded significantly more strongly than the C/EBP mutant to 500 pg of cotransfected C/EBPβ expression vector ($p=0.002$), and to 2.5 ng/ml IL6 ($p=0.01$) (right panel). Normalized luciferase activities are expressed in relation to baseline activity of the wild-type promoter constructs, and means (±SD) of triplicate transfections are shown.

Subsequently, we examined whether an intact C/EBP site at position -133/-125 is necessary for the response of the gene to HNF-3β. In these experiments, pGL3-basic-derived constructs including 400 bp of the proximal fibrinogen β promoter were used, and these constructs were already available at our institute [16]. In the presence of 1000 pg of HNF-3β expression vector, the activity of the wild-type fibrinogen β promoter increased 2-fold, whereas the activity of the fibrinogen β promoter with the mutated C/EBP site was barely induced by HNF-3β (Figure 4b, left panel). Similar results were obtained at lower HNF-3β concentrations. This shows that the HNF-3 response of the fibrinogen β promoter requires a functional C/EBP site at -133/-125. To summarize, these experiments show that the C/EBPβ and HNF-3 sites depend on each other for maximal activity.

Finally, additional transfection assays were performed to confirm the previously reported requirement of the C/EBP site for the IL6 response of the fibrinogen β promoter [14,15]. The activity of the wild-type promoter increased 5.5-fold upon cotransfection of 500 pg of C/EBPβ expression vectors and 4-fold in response to 2.5 ng/ml IL6 (Figure 4b, right panel). The promoter with the mutated C/EBP site however, responded to C/EBPβ with an increase in activity of only 2.5-fold and did not respond to 2.5 ng/ml IL6, underlining the important role of the C/EBP site in the IL6 response of the fibrinogen β gene.

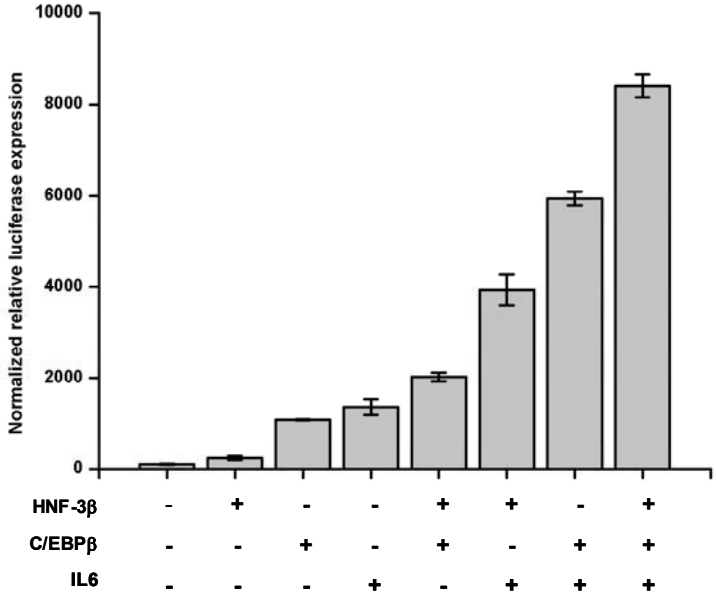


Figure 5: HNF-3β, C/EBPβ and IL6 synergistically enhance fibrinogen β promoter activity. Reporter gene constructs including 400 bp of the wild-type fibrinogen β promoter were transfected into HepG2 cells, in the presence or absence of 500 pg of HNF-3β expression vector, and/ or 250 pg of C/EBPβ expression vector, and/ or 2 ng/ml IL6. Normalized luciferase activities are expressed in relation to baseline activity and means (±SD) of triplicate transfections are shown

Synergistic effects of HNF-3β, interleukin-6 and C/EBPβ on fibrinogen β promoter activity

To study the combined effect of HNF-3β, IL6 and C/EBPβ on the fibrinogen β promoter, the pGL3-basic constructs including 400 bp of the wild-type fibrinogen β promoter were used [16]. The activity of the fibrinogen promoter increased 2.5-fold in the presence of 500 pg vectors expressing HNF-3β, and increased 13-fold in response to 2 ng/ml IL6 (Figure 5). However, when HNF-3β and IL6 were added simultaneously, the activity of the fibrinogen β promoter increased 40-fold, showing that HNF-3β and IL6 synergistically enhance fibrinogen β promoter activity. For C/EBPβ and IL6 a similar effect

was observed. The addition of C/EBPβ alone (250 pg expression vector) resulted in a 10-fold increase of promoter activity, but when added together with IL6, fibrinogen β promoter activity increased 60-fold. Also for HNF-3β and C/EBPβ a synergistic effect was observed, as the addition of HNF-3β and C/EBPβ together increased promoter activity 20-fold. Finally, when HNF-3β, C/EBPβ and IL6 were all present, fibrinogen β promoter activity increased 85-fold compared to the basal expression level. Synergistic stimulation of IL6-induced fibrinogen β promoter activity indicates that both C/EBPβ and HNF-3β are important components of the transcriptional complex regulating the IL6 response of the fibrinogen β gene.

DISCUSSION

In this study, we identified a new functional HNF-3 site at –159/-151 in the fibrinogen β promoter and we demonstrated its involvement in the IL6 response of the gene. The transcription factor C/EBPβ, which is activated by IL6, plays a central role in the IL6 induction of the fibrinogen β gene (reviewed by Poli [17]). We observed that the integrity of the HNF-3 site identified is required in order to obtain full response of the promoter to C/EBPβ, which could explain the importance of the HNF-3 site in the IL6 response of the fibrinogen β promoter. In addition, we observed that C/EBPβ and HNF-3β synergistically activate the fibrinogen β promoter. Our results indicate that HNF-3 is most likely part of the transcriptional complex controlling the IL6 response of the fibrinogen β promoter.

Because of our interest in the inflammatory response of the fibrinogen β gene, we have focused on the HNF-3 site adjacent to IL6 responsive sequences. However, there may be other functional HNF-3 sites present in the fibrinogen β promoter that have not been identified yet. Our initial *in silico* promoter analysis did indeed reveal several possible HNF-3 binding motifs and our experimental results also imply the presence of a functional HNF-3 element beside the site at -159/-151. We observed that the long (1800 bp) fibrinogen β promoter fragments responded much more strongly to overexpression of HNF-3 than the short (400 bp) promoter fragments, which could be explained by an additional HNF-3 site in the long fragment (compare Figure 1 and Figure 4b). Secondly, the response of the promoter to HNF-3 was strongly reduced by the mutation of the HNF-3 site at -159/-151, but not completely abolished. This is again an indication of the presence of an additional HNF-3 element further upstream in the fibrinogen β promoter. In theory, this latter observation might also be explained by a reduced but not completely abolished affinity of the HNF-3 site by mutation. However, this hypothesis is counteracted by our results from EMSA experiments, showing that after the introduction of this mutation, no residual binding of proteins to the oligonucleotide is left,

indicating that the HNF-3 site was indeed completely disrupted by the mutation we created. Therefore, our results indicate the presence of an additional HNF-3 site present between -400 and -1800 bp in the fibrinogen β promoter.

Functional C/EBPβ-binding motifs have been characterized in the promoters of numerous acute-phase genes, including haptoglobin, C-reactive protein, serum amyloid A, fibrinogen α and fibrinogen β. The activity of C/EBPβ is mainly regulated by its phosphorylation status, and interleukin-6 is able to activate C/EBPβ by phosphorylation. Phosphorylation of C/EBPβ leads to increased transactivating potency, thus conferring early acute phase signals to the promoters of C/EBP-regulated acute phase genes (reviewed by Poli [17]). The HNF-3 transcription factors (HNF-3α, -3β, and -3γ) are regulators of genes important in inflammation and development, and in contrast to C/EBP, HNF-3 expression is regulated at the transcriptional level. Cytokine-responsive C/EBPβ and C/EBPδ proteins induce the HNF-3β promoter upon activation by cytokines, and can pass delayed interleukin-6 and interleukin-1 stimulation on to HNF-3β responsive promoters [22]. It has been shown *in vitro* that HNF-3, but not C/EBP, can bind to its binding site in compacted chromatin and open the local nucleosomal domain. The ability of HNF-3 to open chromatin is mediated by a high affinity DNA-binding site, and a C-terminal domain of the protein which binds histones [23]. The synergistic enhancement of fibrinogen β promoter activity by HNF-3 and C/EBP that we observed in our experiments, suggests that HNF-3 and C/EBP cooperate. The different mechanisms of promoter activation by HNF-3β and C/EBPβ, briefly described above, may provide a hypothesis for this cooperation. HNF-3 could make the IL6 responsive sequences in the DNA accessible for IL6-activated transcription factors such as C/EBPβ, which then activate the promoter. Cooperation between HNF-3 and C/EBP is in line with previous reports, in which synergistic enhancement of target promoters by HNF-3 and C/EBPβ has been described [24]. In addition, HNF-3 has also been shown to synergize with other transcription factors including STAT3 and p53 [25,26].

In this study we show that mutations in the HNF-3 binding sequence 8 bp upstream of the IL6 RE result in a strongly decreased IL6 response, and we confirm that mutations in the C/EBP element located 4 bp downstream of the IL6 RE also lead to a reduced IL6 response. In addition, a common C to T polymorphism is located at position -148, three base pairs downstream of the HNF-3 binding site, and there is evidence that this polymorphism affects fibrinogen β promoter activity [27]. Between the HNF-3 site at position –159/ -151 and the C/EBP site at position –143/-137, an IL6 RE is located (Table 1). Mutations in this IL6 RE result in loss of IL6 response of the fibrinogen β promoter [14-16]. This shows that the entire highly conserved DNA sequence from -160 to -125 in the fibrinogen β promoter is involved in the regulation of fibrinogen β gene expression, and more specifically in the IL6 response of the

gene. So far, no transcription factor binding to the IL6 RE has been identified. The IL6 RE may bind a transcription factor of its own, or it may be required for a correct positioning of the HNF-3 and C/EBP sites in relation to each other, or both. The fact that a possible third transcription factor has never been found might be explained by the fact that this factor may only bind in the simultaneous presence of HNF-3 and C/EBPβ. Since EMSA experiments are usually performed with an excess of oligonucleotide, the complex containing the three transcription factors will not be formed in such experiments.

The DNA sequence of the IL6 RE in the human fibrinogen β promoter is identical to the sequence of the three IL6 REs in the human fibrinogen γ promoter, and it has been shown recently that signal transducer and activator of transcription-3 (STAT3) binds to each of the three IL6 REs in the fibrinogen γ promoter [28]. Therefore STAT3 could be an interesting candidate for binding to the IL6 RE in the fibrinogen β promoter, and similar cooperative interaction of different sites such as we at present propose for the fibrinogen β promoter may also be important for the fibrinogen γ promoter.

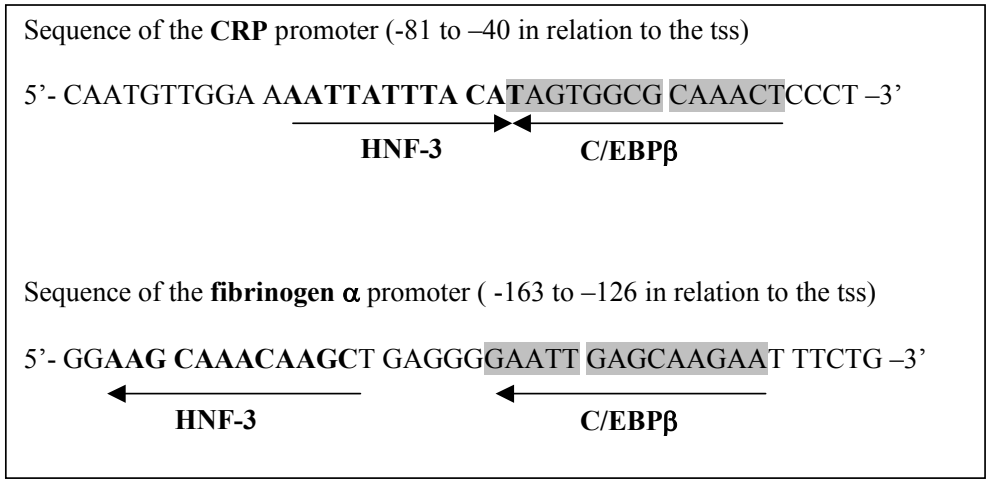


Figure 6: Putative HNF-3 sites in the fibrinogen α and C-reactive protein promoters. The fibrinogen α promoter and C-reactive protein promoter sequences include putative HNF-3 sites adjacent to cytokine-responsive C/EBPβ sites [29,30].

The role of HNF-3 in the IL6-regulated gene response that we describe, could be shared by other acute phase genes. Indeed, inspection of promoter regions of several other acute phase genes revealed that the C-reactive protein promoter and the fibrinogen α promoter have putative HNF-3 sites located within a few base pairs from cytokine responsive C/EBPβ elements [29,30] (Figure 6), indicating that the involvement of HNF-3 in the IL6 response may in fact be a more general regulatory mechanism of the hepatic acute phase response.

To summarize, we have identified a functional HNF-3 site at position –159/-151 in the fibrinogen β promoter that is important for interleukin-6-induced fibrinogen β promoter activity. The necessity of HNF-3 in the IL6 response can be explained by the dependency of the adjacent IL6-responsive C/EBP site on this HNF-3 site. This work contributes to the understanding of the regulation of the hepatic acute-phase response of fibrinogen β, and it demonstrates a new function for HNF-3.

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Chapter 3

The fibrinogen β -148C/T promoter polymorphism modulates the response of the gene to interleukin-6 by influencing the activity of the adjacent hepatocyte nuclear factor-3 (HNF-3) site

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Submitted (combined with chapter 2)

The fibrinogen β -148C/T promoter polymorphism modulates the response of the gene to interleukin-6 by influencing the activity of the adjacent hepatocyte nuclear factor-3 (HNF-3) site

Maartje Verschuur, Maureen de Jong, Hans L. Vos and Moniek P.M. de Maat

ABSTRACT

Elevated fibrinogen levels are an important risk factor for the development of cardiovascular disease, and genetic variation in the fibrinogen β gene partially determines plasma fibrinogen levels. The A allele of the -455G/A polymorphism is associated with elevated habitual plasma fibrinogen levels and with a stronger inflammatory response of fibrinogen levels. However, it is unclear whether the -455G/A polymorphism is causal, or just a marker of the functional allele, which is characterized by -1420A, -993T, -455A and -148T in Caucasians. The aim of our study was to identify the functional variation in the fibrinogen β promoter and to elucidate the molecular mechanism. Luciferase reporter gene assays in hepatoma cells revealed that only the transition from C to T at -148 affected promoter activity. It resulted in a decrease of ± 30% in basal expression and a decrease of ± 50% in IL6-induced expression, and the differences between the two alleles was highly significant. We show with cotransfection experiments and gel-shift assays that the -148C/T polymorphism influences the HNF-3 site at -159/-151. It has been shown that the functionality of the binding site for the (IL6-activatable) transcription factor C/EBPβ depends on the integrity of this HNF-3 site, and this explains the influence of the -148C/T variation on fibrinogen β promoter activity. In conclusion, we provide evidence that the -148C/T variation in the fibrinogen β promoter is functional, and therefore we suggest that -148C/T should be determined in epidemiological studies.

INTRODUCTION

Epidemiological studies have consistently shown that an elevated plasma fibrinogen level is a risk factor for cardiovascular disease [1,2]. Elevated plasma fibrinogen levels are associated with an increased risk of ischaemic heart disease [1,3], and fibrinogen is found in atherosclerotic plaques [4]. Furthermore, an increased plasma fibrinogen level is associated with an increased risk of venous thrombosis [5]. Therefore, much interest has been shown in factors that determine fibrinogen levels.

Human fibrinogen is composed of 3 pairs of polypeptide chains: two α chains, two β chains and two γ chains. Each of these proteins is encoded by a separate gene, and the three fibrinogen genes together form a 50 kb cluster on the long arm of chromosome 4. Fibrinogen is synthesized in the liver and its expression is mainly regulated at the level of transcription. There is evidence that in humans transcription of the gene encoding the β chain is rate-limiting [6]. Fibrinogen is a strong acute-phase reactant, and inflammatory stimuli such as strenuous exercise, smoking and trauma result in increased fibrinogen levels [7-12]. The main inducer of fibrinogen in the acute-phase response is interleukin-6 (IL6) [13-16]. Several papers have been published describing the molecular mechanism of the acute phase response of fibrinogen. The IL6 response of the fibrinogen β promoter is mediated by an IL6-responsive element (IL6 RE) located at -143/-137, a C/EBPβ binding site located at –133/-125 [17-19], and also an HNF-3 site located at –159/-151, as we have recently demonstrated (**chapter 2**).

The relationship between genetic variation of the fibrinogen β chain and plasma fibrinogen levels has been studied on many occasions. The A allele of the fibrinogen β -455G/A variation has consistently been associated with elevated habitual fibrinogen levels [20-25], and an interaction between the -455G/A polymorphism and acute-phase stimuli has been reported repeatedly as well (reviewed by Humphries *et al* [26]). Subjects with at least one A allele respond to trauma, surgery or strenuous exercise with a stronger rise in fibrinogen levels than -455GG homozygotes, and in most of these reports a critical role is attributed to the inflammatory mediator IL6 [27-30]. Besides the -455G/A polymorphism, there are several other polymorphisms in the promoter of the fibrinogen β gene. In the promoter region, the -1420G/A, -993C/T and -148C/T variations are in complete linkage disequilibrium with -455G/A in Caucasians [31,32]. Thus Caucasian carriers of the -455A allele also have -1420A, -993T and -148T, and any of these variations therefore could influence fibrinogen levels. Knowing the functional variation(s) is important from an epidemiological point of view, as these polymorphisms are not always coinherited in other ethnic groups [33]. Furthermore, identifying the functional variations and understanding the mechanism are essential for insight into the variation between individuals with respect to their response to inflammation.

The first aim of our study was to assess the contribution of genetic variation of the fibrinogen β promoter to IL6-induced and basal fibrinogen β promoter activity, and to identify the functional variant(s). The second aim of this study was to elucidate the molecular mechanism responsible. Our results show that the -148C/T variation in the fibrinogen β promoter is functional both under IL6-induced and basal conditions. The -148C/T variation influences the response of the promoter to HNF-3 and C/EBPβ, providing a molecular explanation for the functional effects of this polymorphism.

METHODS

Materials

CELL LINES

The human hepatoma cell lines HepG2 (American Type Culture Collection) and HuH7 [34] were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Whittaker Europe) and 10% foetal bovine serum (FBS, Invitrogen).

PGL3-FIBRINOGEN β PROMOTER REPORTER GENE CONSTRUCTS

1800 bp of the promoter region of the fibrinogen β gene (-1788 to +8, taking position 1500 in Genbank accession number X05018 as +1, the transcription start site) was amplified by PCR on the genomic DNA of individuals carrying different alleles of the -455G/A polymorphism. In this PCR reaction 200 μM of each dNTP, 10 pmol of each primer (forward primer: 5'-TCT TAC GCG TGA AGA ATG CCA ATC AGA GTA-3', reverse primer: 5'-TCA TCT CGA GTA GAC TTA ACT GAG AGA TCT TCA-3'), 3.5 U of High Fidelity polymerase (Roche) and 50 ng of genomic DNA in a total volume of 50 μl was used. The PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 4 min, and a final extension at 72°C for 5 min. PCR products were digested with *Mlu*I and *Xho*I (the introduced restriction sites are underlined in the primer sequences) and cloned into the *Mlu*I and *Xho*I sites of pGL3-basic (Promega), upstream from the firefly luciferase gene. Finally, four pGL3-basic constructs each containing 1800 bp of the fibrinogen β promoter with a different promoter haplotype were obtained: pGL3-FGB haplo a, pGL3-FGB haplo b, pGL3-FGB haplo c, and pGL3-FGB haplo d (Table 1). To be able to discriminate between the effects of the variants unique to haplotype d (-1420A, -993T, -455A, -148T), artificial promoter haplotypes were constructed. By exchanging the *Bsa*BI-*Mlu*I restriction fragment (which spans the region from -1788 to -269 relative to the transcription start site) between pGL3-FGB haplo b and pGL3-FGB haplo d, two additional constructs (pGL3-FGB haplo b'-

148T and pGL3-FGB haplo d'-148C) were derived (Table 1). All constructs were sequenced to verify the identity of the polymorphic sites and to make sure that no PCR artefacts had been introduced. In addition, the identity of the fibrinogen β promoter haplotype constructs was confirmed on several occasions between transfection experiments. For this, restriction digests were used, as the change from G to A at -455 abolishes a *Hae*III site and the change from C to T at -148 abolishes a *Hind*III site.

VECTORS EXPRESSING TRANSCRIPTION FACTORS

Vectors expressing the three HNF-3 isoforms and C/EBPβ (*pCDNA3.1-HNF3α, pCDNA3.1-HNF3β, pCDNA3.1-HNF3γ, pSCT-C/EBPβ*, all from rat origin) and their empty counterparts (*pCDNA3.1-, pSCT-*), were a kind gift from Dr. P. Holthuizen (Department of Physiological Chemistry, University of Utrecht, the Netherlands).

Table 1: Fibrinogen β promoter haplotypes present in pGL3-basic and their frequencies in the Caucasian population.

	-1420 G/A	-993 C/T	-854 G/A	-455 G/A	-249 C/T	-148 C/T	Frequency
Natural haplotypes							
Haplotype a	G	C	G	G	C	C	≈ 0.40
Haplotype b	G	C	**A**	G	C	C	≈ 0.20
Haplotype c	G	C	G	G	**T**	C	≈ 0.20
Haplotype d	**A**	**T**	G	**A**	C	**T**	≈ 0.20
Constructed haplotypes							
Haplotype b'-148T	G	C	**A**	G	C	**T**	
Haplotype d'-148C	**A**	**T**	G	**A**	C	**C**	

Fibrinogen β promoter haplotypes are present as 1800 bp promoter fragment in pGL3-basic and frequency data are as reported [43]. For easy comparison with the constructed haplotypes, in some of the figures the natural haplotype b is also called haplotype b-148C, and the natural haplotype d is also called haplotype d-148T.

Luciferase-reporter gene assays

TRANSFECTION CONDITIONS

HepG2 and HuH7 cells were plated in 24-wells plates in DMEM with 10% FBS at a density of 1.0 *10^5 cells per well. After the cells had been allowed to attach overnight, the medium was replaced with serum-free medium supplemented with 0.1% human serum albumin (HSA, Cealb®). After 2 h, cells were transfected using FuGene 6 (Roche), according to the manufacturer's protocol.

200 ng of pGL3 construct, and 4 ng of pRL-tk (Renilla luciferase expression construct, Promega), were used per well. If applicable, vector expressing HNF-3α, HNF-3β, HNF-3γ or C/EBPβ, or a molar equivalent of the empty expression vector as control were added. The total amount of DNA was kept at 400 ng per well with carrier DNA (herring sperm, Invitrogen). The effect of the empty expression vectors on fibrinogen β promoter activity and on the Renilla luciferase expression was determined for all conditions tested in this study, and no effects of the empty control vectors were detected. For each construct at least two independent DNA preparations were used, and all DNA preparations were transfected at least twice in triplicate. 24 hrs after transfection, the medium was replaced with DMEM + 0.1% HSA, containing IL6 concentrations ranging from 0 to 15 ng/ml (recombinant human IL6, Pepro Tech). In healthy subjects systemic IL6 levels up to 0.1 ng/ml can be found and in subjects with signs of severe inflammation IL6 levels up to 1.4 ng/ml have been reported [15,35-40]. IL6 concentrations in both the physiological and supra-physiological ranges were used.

LUCIFERASE ASSAY

After the cells had been cultured for 24 hrs in the presence of IL6, they were washed once with 500 µl phosphate-buffered saline (PBS) and subsequently lysed for 15 minutes on a rotary platform at room temperature with 100 µl Passive Lysis Buffer (PLB, Promega). The Firefly luciferase reporter and Renilla luciferase internal control activities were measured in 10 µl lysate, using the Dual-Luciferase® Reporter Assay System (Promega). Luminescence was measured using a luminometer (Berthold).

Electrophoretic Mobility Shift Assay (EMSA)

PREPARATION OF NUCLEAR EXTRACTS

HepG2 cells were cultured under serum-free conditions (DMEM P/S + 0.1% HSA) for 24h prior to the preparation of the nuclear extracts. For the preparation of nuclear extracts of IL6-stimulated cells, cells were incubated with 5 ng/ml IL6 for 15 minutes. Cells were washed and lysed, and nuclear extracts were prepared according to the method of Slomiany *et al* [41]. Buffers were supplemented with protease inhibitor cocktail (Complete™ Mini, Roche), and with phosphatase inhibitors (Na-orthovanadate, final concentration 250 µM; β-glycerophosphate, final concentration 25 mM). The protein concentration in the nuclear extracts was estimated using the BCA micro kit (Pierce), and the samples were stored at –80°C for future use.

OLIGONUCLEOTIDES

Double-stranded 26 bp oligonucleotides were designed surrounding the -148C/T polymorphism and representing either the C or the T allele of the

-148C/T polymorphism, and included the HNF-3 site at –159/-151 (Table 2). Pairs of complementary oligonucleotides were annealed at equimolar amounts and radioactively labelled at the 5' ends with γ-^{32}ATP using T4 polynucleotide kinase (Invitrogen). The double-stranded and labelled oligonucleotides were purified using MicroSpin G-25 Columns (Amersham Pharmacia Biotech), and stored at –20°C for future use.

Table 2: Sequence of the oligonucleotides used in the EMSAs

Fibrinogen β promoter sequence
5' TATGACAAGTAAATAAG**C**TTTG**CTGGGAA**GAT**GTTGCTTAA**ATGA 3'
3' ATACTG**TTCATTTAT**TC**G**AAACGACCCTTCTACAACGAATTTACT 5'
HNF-3 IL6 RE C/EBP
Fibrinogen β -148 C oligo
5'- TATGACAAGTAAATAAG**C**TTTGCTGG -3'
Fibrinogen β -148 T oligo
5'- TATGACAAGTAAATAAG**T**TTTGCTGG -3'

BINDING REACTION AND ELECTROPHORESIS

For each binding reaction, 0.5-4 μg of nuclear extract was pre-incubated for 30 minutes on ice with 5 μg poly dIdC (Amersham Pharmacia Biotech) in a 12 μl reaction mixture containing 10 mM Tris/HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol, 250 μM Na-orthovanadate and 25 mM β-glycerophosphate. Subsequently, the labelled oligonucleotides were added to the pre-binding reactions, and this mixture was incubated for another 30 minutes on ice. The reaction products were loaded onto 5% non-denaturing polyacrylamide gels and run in 0.25x TBE (1x TBE = 0.1M Tris, 0.09M Boric Acid, 0.001M EDTA). Gels were blotted on Whatmann paper and exposed to autoradiograph film overnight at -80°C with intensifying screens.

Statistical methods

Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity as an internal standard. These normalized luciferase activity levels were expressed as a percentage of the normalized luciferase activity of pGL3-FGB haplo d or pGL3-FGB haplo b'-148T at baseline. Normalized expression levels of the different haplotype constructs were compared by ANOVA testing followed by LSD post hoc testing, or with

Student's *t*-test; SPSS 11.0 for Windows was used. P-values are described in the results section or otherwise stated in the figure legends.

RESULTS

Identification of the functional polymorphism in the fibrinogen β promoter under IL6-induced conditions

1800 bp fragments of the four common fibrinogen β promoter haplotypes were cloned into the luciferase expression vector pGL3-basic and sequenced (Table 1). During this process the known polymorphisms in the fibrinogen β promoter were confirmed and no new polymorphisms were detected. IL6 treatment of HepG2 cells transfected with the constructs with the four natural promoter haplotypes resulted in a strong induction (up to 25-fold) of all four fibrinogen β promoter variants (Figure 1).

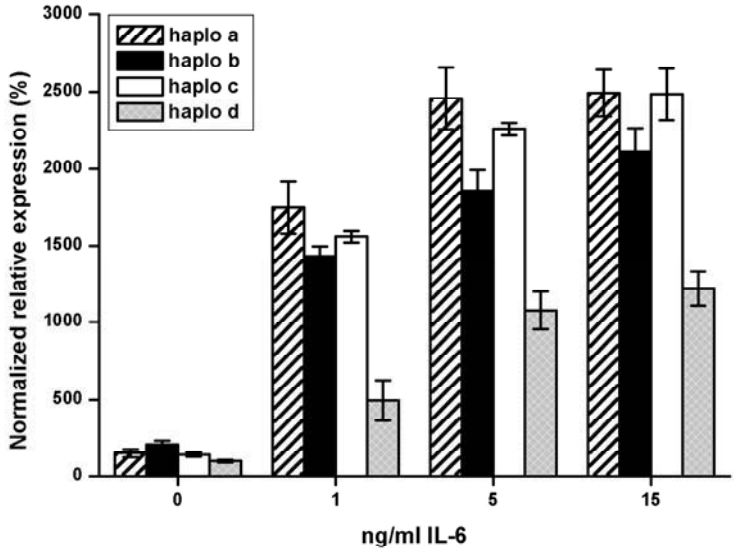

Figure 1: IL6-induced expression of natural fibrinogen β promoter haplotypes in HepG2 cells. Fibrinogen β promoter constructs were transfected into HepG2 cells, and cells were subsequently treated with IL6. The IL6-induced promoter activity of haplotype d was significantly lower than the IL6-induced promoter activity of the other haplotypes ($p<0.001$ at all IL6 concentrations). Normalized luciferase activities are expressed in relation to baseline activity of the haplotype d construct and means (±SD) of triplicate transfections are shown.

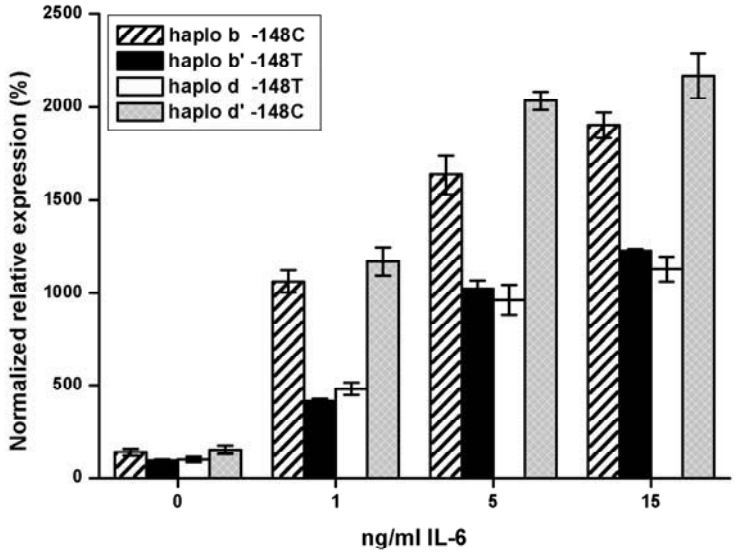

Figure 2: IL6-induced expression of constructed fibrinogen β promoter haplotypes in HepG2 cells and identification of the functional polymorphism. Fibrinogen β promoter constructs were transfected into HepG2 cells, and cells were subsequently treated with IL6. The IL6-induced promoter activity of the haplotypes d-148T and b'-148T was significantly lower than the IL6-induced promoter activity of the haplotypes with C at position -148 (haplotype b-148C and d'-148C) ($p<0.001$ at all IL6 concentrations). Normalized luciferase activities are expressed in relation to baseline activity of the haplotype d construct and means (±SD) of triplicate transfections are shown.

However, the IL6-induced expression of promoter haplotype d was approximately 50% lower than the IL6-induced expression of haplotypes a, b and c. In addition, when the response of the promoter constructs to IL6 were expressed in relation to the baseline activity of the individual construct (as 'fold induction'), also this relative induction haplotype d was substantially lower (35%-50%) than the relative induction of haplotypes a, b and c (data not shown).

The -1420A, -993T, -455A and -148T alleles are all unique to haplotype d, and this implies that any of these base pair substitutions (or a specific combination) could be responsible for the haplotype-dependent difference in the response to IL6 observed. The -148C/T polymorphism is particularly interesting, as it is located between the HNF-3β site, and the IL6 RE and C/EBPβ site [17-19]. To investigate the functionality of the -148C/T variation, artificial haplotypes that differed only at -148C/T from their natural counterparts were used in transfection experiments in hepatoma cells. IL6 treatment of HepG2 cells transfected with the artificial fibrinogen β promoter variants again resulted in a strong induction of these fibrinogen β promoter variants (Figure 2). The IL6-induced expression of haplotypes b-148C and d'-148C was significantly higher

than the IL6-induced expression of haplotypes d-148T and b'-148T. In addition, when the response of the promoter constructs to IL6 were expressed in relation to the baseline activity of the individual construct (as 'fold induction'), also this relative induction of constructs carrying T at position -148 was substantially lower (35%-50%) than the relative induction of haplotypes with a C at position -148 (data not shown). The approximately 50% lower IL6 induced promoter activity of haplotypes d-148T and b'-148T was confirmed in HuH7 cells, but the IL6 response of the fibrinogen β promoter variants was much smaller in HuH7 cells than in HepG2 cells (data not shown). These results show that transition from C to T at -148 resulted in a strong decrease in IL6-induced expression, and *vice versa*. There were no significant differences between the activity of constructs carrying the same -148 allele, but differing at all other polymorphic sites (between haplotype b-148C and d'-148C, and between haplotype d-148T and b'-148T). This indicates that it is the -148C/T polymorphism that is the main determinant of the lowered IL6 response of haplotype d.

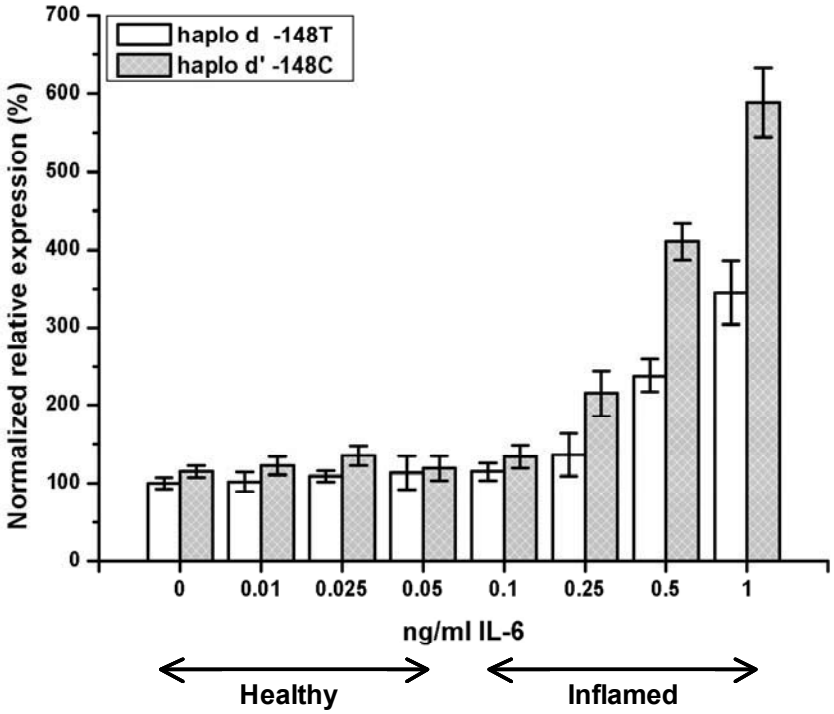

Figure 3: The response of fibrinogen β promoter haplotypes to physiological IL6 concentrations. Fibrinogen β promoter constructs were transfected into HepG2 cells, and cells were subsequently treated with concentrations IL6 that occur *in vivo*. The IL6-induced promoter activity of haplotypes d-148T was significantly lower than the IL6-induced promoter activity of haplotype d'-148C at 0.5 ng/ml IL6 (p=0.001) and at 1.0 ng/ml (p=0.002). Normalized luciferase activities are expressed in relation to baseline activity of the haplotype d construct and means (±SD) of triplicate transfections are shown.

The significance of the –148C/T polymorphism under physiological conditions was verified in an additional experiment in which low IL6 levels, as they occur systemically *in vivo*, were used. The fibrinogen β promoter responded to IL6 concentrations as low as 0.25 ng/ml, and the difference in IL6-induced expression between the two promoter variants was already significant at these physiological IL6 concentrations (Figure 3).

The -148C/T polymorphism influences basal fibrinogen β promoter activity
In addition, the effect of the -148C/T variation on basal expression of the fibrinogen β promoter was determined. For this, the results from all experiments were pooled and analyzed together. This analysis showed that the basal expression levels of each individual construct with a C at -148 (haplotypes a, b and c and d'-148C) was approximately 30% higher than the basal expression levels of each individual construct with a T at -148 (haplotypes d and b'-148T) (Figure 4).

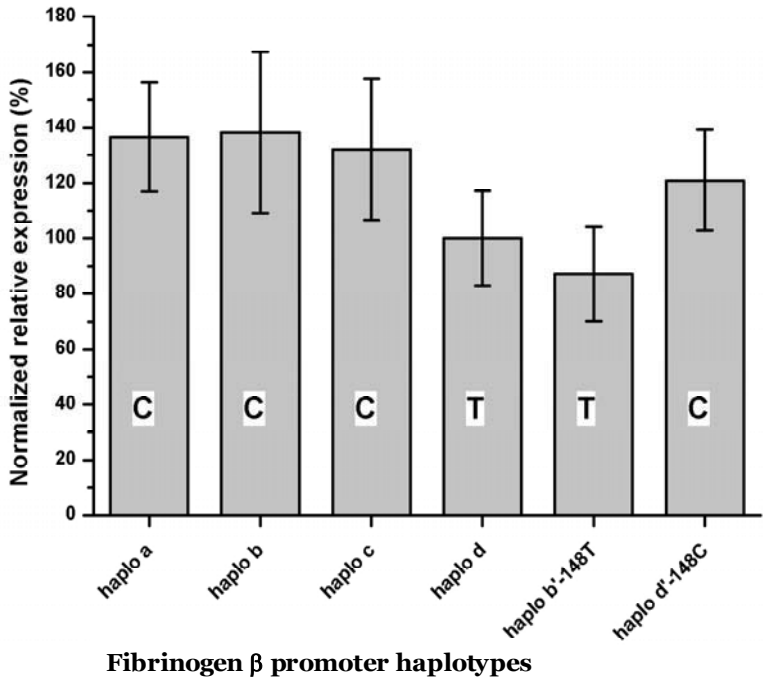

Figure 4: Basal expression levels of fibrinogen β promoter haplotypes in HepG2 cells. Fibrinogen β promoter constructs were transfected into HepG2 cells. Basal promoter activity of the haplotypes d and b'148 was significantly lower than the basal promoter activity of all other haplotypes ($p<0.01$ in all cases). Normalized luciferase activities are expressed in relation to the activity of the haplotype d construct. Means (±SD) of 12-24 transfections obtained in 7 independent experiments of triplicate transfections are shown. The labels in the bars indicate the nucleotide present at position -148.

However, in individual experiments, the effect of the -148C/T polymorphism on basal promoter activity was sometimes less clear. There were no significant differences in basal expression levels between any of the constructs with a C at position -148, or between those with a T at position -148.

HNF-3β binds preferentially to the –148C allele under basal and IL6-induced conditions

The electrophoretic mobility shift assays (EMSAs) described in **chapter 2** had shown that HNF-3β binds to the HNF-3 site 3 bp upstream of the –148C/T polymorphism. Therefore, EMSAs designed to evaluate the affinity of the -148C and -148T alleles for HNF-3β were performed.

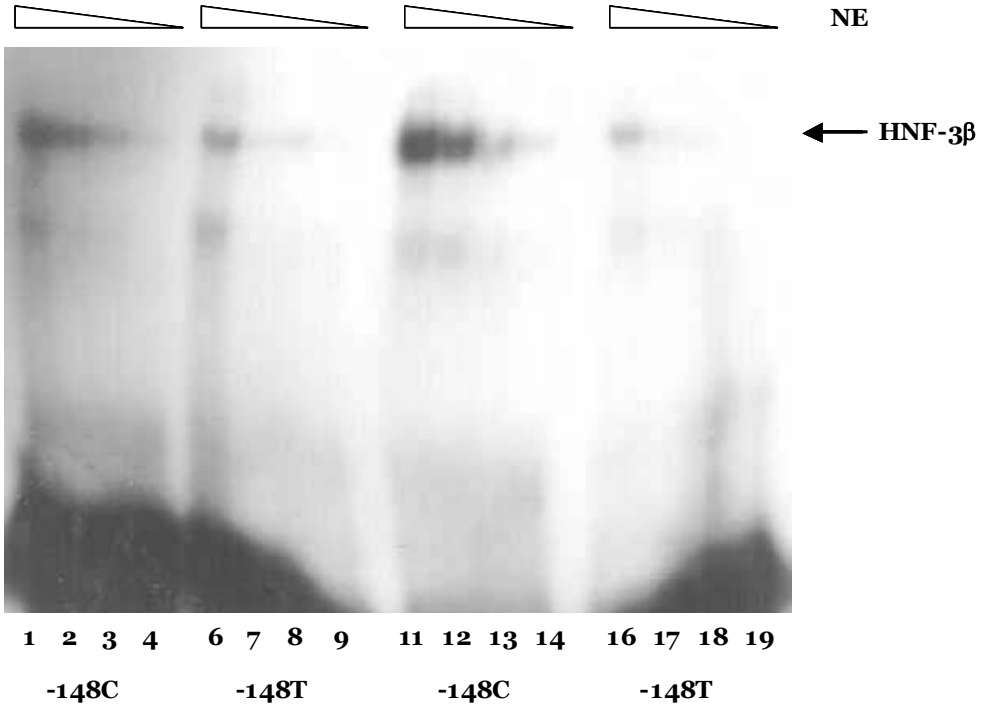

Figure 5: The -148C/T polymorphism influences binding of HNF-3β to the fibrinogen β promoter. Labelled oligonucleotides representing the -148C allele (lanes 1 to 4 and 11 to 14), or the -148T allele (lanes 6 to 9 and 16 to 19) were incubated with decreasing amounts of nuclear extracts (lanes 1, 6, 11, 16 all 4 μg, lanes 2, 7, 12, 17 all 2 μg, lanes 3, 8, 13, 18 all 1 μg, and lanes 4, 9, 14, 19 all 0.5 μg). Nuclear extracts derived from HepG2 cells cultured under basal conditions (lanes 1 to 9) and from HepG2 cells cultured under IL6-induced conditions (lanes 11 to 19) were used.

A major complex, in **chapter 2** identified as HNF-3β, bound to both alleles under basal- and IL6-induced conditions. The complex bound to the C allele at all amounts of nuclear extract, but disappeared from the T allele when less than 4 μg nuclear extract was used (Figure 5). This might suggest that HNF-3β binds

preferentially to the C allele of the –148C/T polymorphism. No qualitative differences between the EMSA patterns of basal and IL6-induced conditions were observed.

The –148C/T variation affects the response of the fibrinogen β promoter to HNF-3

We observed that the –148C/T polymorphism influences binding of HNF-3β to the HNF-3 site in the fibrinogen β promoter. Next, we examined whether the -148C/T variation also affects the response of the fibrinogen β promoter to HNF-3, by transfection of fibrinogen β promoter haplotypes b and b'-148T in the presence of overexpressed HNF-3 isoforms. The -148C allele responded to overexpression of HNF-3α (50-500 pg) with a 2-fold increase in activity, whereas the -148T allele did not respond to HNF-3α (Figure 6).

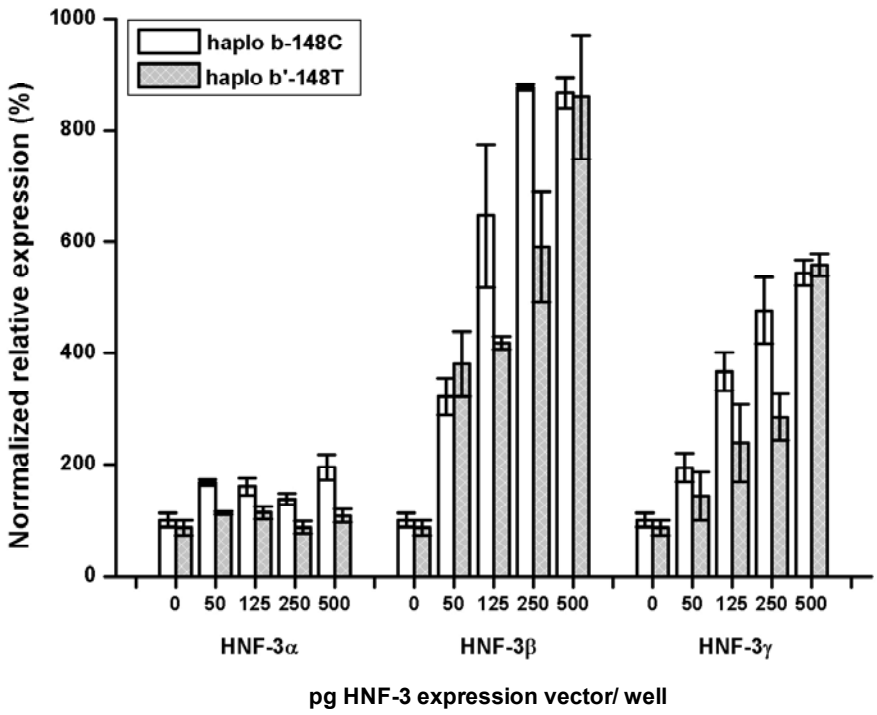

Figure 6: The -148C/T variation influences induction of the fibrinogen β promoter haplotypes by HNF-3 isoforms. Fibrinogen β promoter reporter gene constructs were transfected into HepG2 cells, in the presence of increasing amounts of HNF-3α, HNF-3β or HNF-3γ expression vector. The differences in response to HNF-3 between the alleles were statistically significant at all HNF-3α concentrations ($p<0.01$ in all cases), when 125 or 250 pg of HNF-3β expression vector were cotransfected ($p=0.037$ and $p=0.007$ respectively), and when 125 and 250 pg of vector HNF-3γ expression vector were cotransfected ($p=0.046$ and $p=0.011$ respectively). Normalized luciferase activities are expressed in relation to baseline activity of the haplotype b (-148C) construct. Means (±SD) of triplicate transfections are shown.

The activity of the fibrinogen β promoter increased up to 9-fold in response to overexpression of HNF-3β, and up to 6-fold in response to overexpressed HNF-3γ. The activity of the -148C allele was significantly higher than the activity of the -148T allele when 125 and 250 pg of vector expressing HNF-3β or HNF-3γ were cotransfected. At 50 pg HNF-3β or HNF-3γ, differences between the alleles could not be detected, and at 500 pg HNF-3β or HNF-3γ, the activity of the -148C allele did not increase any more. These results show that a change from C to T at position -148 results in a decreased response of the fibrinogen β promoter to HNF-3 isoforms.

The –148C/T variation affects the response of the fibrinogen β promoter to C/EBPβ

The effect of the -148C/T variation on the response of the fibrinogen β promoter to HNF-3 might be explained by an effect of this polymorphism on the adjacent HNF-3 site. **Chapter 2** demonstrates that the response to the IL6-activatable transcription factor C/EBPβ depends on the HNF-3 site, therefore the effect of the -148C/T variation on the response of the fibrinogen β promoter to overexpressed C/EBPβ was verified.

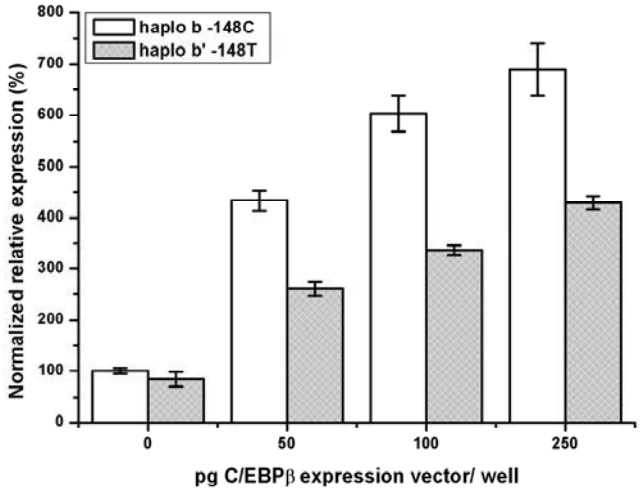

Figure 7: The -148C/T variation influences C/EBPβ-induced activity of the fibrinogen β promoter. Fibrinogen β promoter reporter gene constructs were transfected into HepG2 cells, together with increasing amounts of C/EBPβ expression vector. The C/EBPβ-induced promoter activity of haplotype b-148C was significantly higher than the C/EBPβ-induced promoter activity of haplotype b'-148T ($p<0.0005$ at all C/EBPβ concentrations). Normalized luciferase activities are expressed in relation to baseline activity of the haplotype b (-148C) construct. Means (±SD) of triplicate transfections are shown.

When fibrinogen β promoter constructs differing only at -148C/T (haplotype b and b'-148T) were transfected in the presence of overexpressed C/EBPβ, the activity of the C allele increased up to 7-fold, whereas the activity of the T allele increased only 4-fold (Figure 7). Similar differences were also present at lower C/EBPβ concentrations. Because the HNF-3 and the C/EBP sites interact and are both involved in the IL6 response of the fibrinogen β promoter (**chapter 2**), a lower response of the -148T allele to C/EBPβ and HNF-3 may explain the decreased IL6-induced expression observed for the T allele.

DISCUSSION

In this study we show that the -148C/T variation in the fibrinogen β promoter is a strong determinant of both basal and IL6-induced promoter activity. We observed no indications of a functional role for any of the other promoter polymorphisms, since the -148C/T variation was able to explain the entire difference between haplotype d and the other haplotypes in our assay system. As the -148C/T variation significantly influenced basal promoter activity, and promoter activity under mild (physiological) and strong IL6-induced conditions, an effect of -148C/T on fibrinogen β expression *in vivo* is plausible. In addition, we provide evidence that the -148C/T variation influences the adjacent HNF-3 site, which can explain the functionality of the -148C/T variation at a molecular level.

The -148C/T polymorphism is located in an evolutionarily conserved region of the fibrinogen β promoter. It is located 3 bp downstream of the HNF-3 site, 5 bp upstream of the IL6 RE and 15 bp upstream of the C/EBPβ site [18,19]. C/EBPβ is activated by phosphorylation upon IL6 stimulation, then translocates to the nucleus, binds to the C/EBP site in the promoter and induces promoter activity (reviewed by Poli [42]). Binding of C/EBPα and C/EBPβ to the C/EBP site in the fibrinogen β promoter has been demonstrated [18]. The C/EBP site in the fibrinogen β promoter is necessary for the response of the fibrinogen β promoter to IL6, and it has been shown that mutation of the C/EBP site results in a moderate reduction of basal promoter activity [17-19]. The HNF-3β site at -159/-151 has been discovered recently and the activity mediated by the C/EBP site depends on the integrity of the HNF-3 site. Mutation of the HNF-3 core sequence results in a diminished response of the fibrinogen β promoter to IL6, which can be explained by an interaction between the C/EBP site and the HNF-3 site (**chapter 2**). The results from the present study are in line with this interaction. Change from C to T at -148 resulted in a possibly weaker binding of HNF-3β to the HNF-3 site, a decreased responsiveness of the fibrinogen β promoter to overexpression of HNF-3, and also a lowered response to overexpressed C/EBPβ. Therefore, the effect of -148C/T on the activity of the adjacent C/EBP and HNF-3 sites provides an explanation for the significantly

lower IL6-induced and basal fibrinogen β promoter activity for the T allele that we observed in our promoter assays.

The identification of -148C/T as the functional variation is important from an epidemiological point of view, as the degree of linkage disequilibrium between the fibrinogen β promoter polymorphisms may differ between ethnic groups. In Caucasians the linkage disequilibrium between -1420G/A, -993C/T, -455G/A and -148C/T is nearly complete, and thus each of the four variations can be used as a marker for the functional -148C/T polymorphism. In other ethnic groups however, the situation can sometimes be different. Cook *et al* showed that the -455G/A and the -148C/T variation were not in complete linkage disequilibrium in an Afro-American population [33]. In this population, a stronger association was seen between -148C/T and plasma fibrinogen levels than between -455G/A and plasma fibrinogen levels. These data support our conclusion that -148C/T is a functional polymorphism, and this implies that determination of -148C/T instead of -455G/A should be used to study the associations with plasma fibrinogen levels in all populations.

In our transfection assays, haplotype d, which includes -148T and -455A, showed weaker IL6-induced and basal expression. Surprisingly, in epidemiological studies, increased basal and increased acute phase-induced fibrinogen levels have generally been associated with -455A, which is contrary to our findings. In this respect, there are some issues that are worth considering.

We performed our studies with both HepG2 and HuH7 cells. Although there are large differences between these cells with respect to the magnitude of their IL6 response, fibrinogen β promoter fragments with haplotype d (-148T) had lower transcriptional activity in both HepG2 and HuH7 cells. A lower transcriptional activity of haplotype d in reporter gene experiments has also been observed in HepG2 and Hep3B cells by another research group [43,44]. Similar results in three different hepatoma cell lines indicate that the discrepancy between the epidemiological reports and our experimental data is not caused by a peculiarity of any of the cell lines used.

In our experiments, we used IL6 to mimic the acute phase response. In the case of an acute-phase event *in vivo* however, besides IL6, several other inflammatory mediators are expressed and IL6 interacts with numerous other inflammatory mediators. In the case of the acute phase response of fibrinogen, interactions between IL6 and interleukin-1β, interleukin-4, interleukin-10 and interleukin-13 are described, underlining the complexity of the inflammatory response of fibrinogen *in vivo* [45,46].

A third possible explanation for the observed discrepancy between our *in vitro* data and the reported *in vivo* associations might involve the IL6 responsive element (IL6 RE). This sequence may only be required for the positioning of the HNF-3 and the C/EBP sites correctly to each other, but it is also possible that this IL6 RE binds a transcription factor of its own. However, the molecular

mechanism of the IL6 RE is still unclear. The HNF-3 site, the IL6 RE and the C/EBP site are all located within a region of only 35 base pairs, and the final output of the IL6 response of the fibrinogen β promoter may depend on a highly sensitive interplay between the transcription factors binding to this region. It is possible that the balance between the transcription factors is somewhat different in our experimental system from *in vivo*. A detailed picture of all the events and transcription factors involved in the regulation of the endogenous fibrinogen β gene, including the IL6 RE, needs to be established before we can better understand the presently observed discrepancy between the *in vivo* and *in vitro* effects.
Another explanation for this apparent discrepancy between our *in vitro* results and the reported *in vivo* data could be the presence of a functional polymorphism located outside the fibrinogen β promoter fragment that we studied, which is responsible for elevated plasma fibrinogen level associated with the -148T allele *in vivo*.

Despite the conclusion that parts of the acute phase regulation of the fibrinogen β gene still have to be elucidated, our results clearly identify -148C/T as a functional variation in the fibrinogen β promoter, and we provide a molecular mechanism. We recommend that the fibrinogen β -148C/T variation is determined in epidemiological surveys.

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Chapter 4

Interindividual variation in the response by fibrinogen, C-reactive protein and interleukin-6 to yellow fever vaccination

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Interindividual variation in the response by fibrinogen, C-reactive protein and interleukin-6 to yellow fever vaccination

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ABSTRACT

The acute phase reaction is important in many disease processes. Habitual levels of the acute phase proteins fibrinogen, C-reactive protein (CRP) and interleukin-6 (IL6) are associated with an increased risk of cardiovascular disease, but the dynamic variation of plasma levels of acute phase proteins may be of importance as well. The aim of this study was to document the variation in response by fibrinogen, CRP and IL6 levels to a mild inflammatory stimulus (yellow-fever vaccination) in 25 healthy individuals. Plasma levels of fibrinogen, CRP and IL6 were determined at baseline and 7 days after vaccination, and genetic polymorphisms in these genes were determined. After vaccination, fibrinogen levels had changed between -13% and +44% (p=0.003), CRP levels between -88% and +672% (NS), and IL6 levels between -55% and +448% (NS). Genetic variation partly explained the interindividual variation in response, as IL6-174G homozygotes showed a significantly stronger increase in CRP levels than IL6-174C allele carriers. In conclusion, this study suggests that a large interindividual variation exists in the acute phase response to yellow fever vaccination, indicating that individuals may be classified as hyper- or hypo-responders, and that genetic variation may influence the responsiveness of an individual.

INTRODUCTION

Inflammation plays an important role in many disease processes. There is a large variation in habitual plasma levels of inflammatory proteins [1]. Increased plasma levels of the acute phase proteins fibrinogen, C-reactive protein (CRP) and interleukin-6 (IL6) have consistently been associated with an increased risk of cardiovascular disease [2-6]. Increased levels of these acute phase proteins may be markers of the progression of atherosclerosis, but there is also evidence that acute phase proteins themselves participate in the development of vascular disease [7,8]. Most studies on the relationship of fibrinogen, CRP and IL6 with cardiovascular risk have focused on basal levels of the acute phase proteins [2-6], but the dynamic variation in plasma levels of these proteins may be just as important. Individuals that respond to small inflammatory triggers with a strong rise in the plasma levels of these acute phase proteins (the hyper-responders) are exposed to these higher levels regularly and they may therefore be at higher cardiovascular risk [9]. Thus, the identification of the hyper- and hypo-responders to inflammatory stimuli could be of clinical relevance.

Genetic variation is one of the factors that could predispose to a stronger response to acute phase triggers. Both population- and functional studies have been published supporting this hypothesis, showing that individuals carrying the A allele of the -455G/A variation in the fibrinogen β gene respond to inflammatory triggers with a stronger rise in fibrinogen plasma levels [10-12]. Similar results have been reported for the effect of the IL6 -174G/C polymorphism on the response of CRP levels. IL6 is an important inducer of CRP expression, and subjects homozygous for the G allele of the -174G/C variation in the IL6 gene respond to inflammation with a stronger elevation of CRP levels [13,14].

In this study, we explored the possibility of identifying individuals as hyper- or hypo-responders, using yellow fever vaccination as a standardized inflammatory stimulus. Vaccination with vaccines that are based on live attenuated viruses such as the yellow fever vaccine can be used as ethically acceptable and standardized inflammatory stimuli. These vaccines cause viraemia a few days after vaccination and viraemia can induce a mild acute phase response with flu-like symptoms [15,16]. The primary aim of this study was to document the interindividual variation in the acute phase response. Secondly, it was investigated whether the fibrinogen β -455G/A and the IL6 -174G/C polymorphisms could influence the response by fibrinogen, CRP and IL6 to an inflammatory trigger. Finally, in this study we evaluated whether an *in vitro* whole-blood bacterial lipo-polysaccharide (LPS) stimulation assay might provide an *in vitro* method for the identification of hypo- and hyperresponders to yellow fever vaccination.

METHODS

Study design

Twenty-five healthy individuals visiting the Vaccination Outpatient Clinic of the Leiden University Medical Center to receive vaccination against yellow fever prior to their travels participated in the study. On day 0, the travellers received 0.5 ml Stamaril Pasteur heat-stable avian leucosis-free 17D yellow fever vaccine subcutaneously in the upper right arm (Pasteur Merieux, MSD). Blood was collected just before and 7 days after the subjects had received the vaccine. A pilot study had shown that fibrinogen and CRP levels peak 7 days after vaccination and that IL6 levels peak after 5 days [17]. All the measurements could be obtained from 22 individuals. Because of our focus on CRP and fibrinogen, the participants were asked to return to the Vaccination Outpatient Clinic at 7 days after vaccination. None of the subjects suffered from liver disease, auto-immune diseases or other immune disorders, acute or chronic infectious diseases, or malignancy. None of the subjects had recently had trauma or surgery, and none of them had received recent vaccination or used medication that could affect the inflammatory response. None of the participants had received yellow fever vaccination before, and no other vaccinations were given simultaneously. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants.

Blood sampling

Blood was collected in tubes containing EDTA (final concentration 1.8 mg/ml, Greiner Labortechnik), and in tubes containing sodium citrate (final concentration 0.105 M, Becton Dickinson). EDTA anti-coagulated blood was used to determine plasma CRP- and IL6 levels, and for the-whole-blood LPS stimulation assay. Sodium citrate anti-coagulated blood was used for fibrinogen determination. Blood samples were either used immediately for the whole-blood LPS stimulation assay, or centrifuged at 4°C, after which plasma was snap-frozen and stored at -80°C until analysis. Buffycoats were collected for DNA isolation. All samples were processed within 1 hour after venepuncture.

Biochemical procedures

Functional fibrinogen was determined according to the chronometrical clotting rate assay by Von Claus (Diagnostica Stago/ Roche Diagnostics). The CRP concentration was determined with an in-house high sensitivity enzyme immunoassay (EIA) [18]. Polyclonal rat anti-human CRP antibodies were used as solid phase capture antibodies, and horseradish peroxidase conjugated polyclonal rabbit anti-human CRP antibodies were used as tagging antibodies (DAKO Diagnostics). Reference and control sera were obtained from Dade-Behring. The IL6 concentration was determined in plasma and in samples of

the whole-blood LPS stimulation assay, with an ultrasensitive EIA for human IL6 (R&D systems).

Fibrinogen β -455G/A and IL6 -174G/C genotyping
Fibrinogen β -455G/A genotype was determined by PCR on genomic DNA, followed by restriction enzyme analysis using *Hae*III, as described previously [19]. IL6 -174G/C genotype was determined by PCR on genomic DNA, followed by restriction enzyme analysis using *Hsp* 92 II, as described previously [13].

***In vitro* whole-blood LPS stimulation assay**
For the whole-blood LPS stimulation assay, blood samples at day 0 were immediately stored at 37°C and the whole-blood stimulation assay was started within 60 minutes after blood sampling. 1 ml blood was added to 1 ml RPMI-1640 culture medium (Bio Whittaker) in a culture plate. LPS was added to a final concentration of 10 ng/ml in duplicate, and the samples were incubated for 24 hours at 37°C. After incubation, samples were centrifuged for 20 minutes at 2000 rpm, plasma was collected, snap-frozen and stored at -80°C for IL6 determination [9,20].

Statistical analysis
Because of the small sample size and because of the skewed distribution of the variables, all statistical analyses were performed using non-parametric methods. Plasma levels before and after vaccination were compared by the Wilcoxon Signed Rank Test, and relative changes in plasma levels between genotype groups were analyzed using the Mann-Whitney Test. The Spearman correlation coefficient was calculated to analyze correlation between variables. Data are presented as median values with the observed range, unless otherwise indicated. Genotype distributions were compared to those expected if the alleles were in Hardy-Weinberg equilibrium using the Chi-squared test. Statistical analyses were performed using SPSS for windows, release 11.0.

RESULTS

Characteristics of the study population at baseline
13 males and 12 females were included in this study, and their median age was 24 years (range 19 - 54). There was a significant correlation between CRP and fibrinogen levels for the whole group at baseline (r=0.54, p=0.006).

Changes in fibrinogen, CRP and IL6 plasma levels after yellow fever vaccination

With respect to the response by the inflammatory markers to yellow fever vaccination, plasma levels of all markers were increased 7 days after vaccination, but this increase was only significant for fibrinogen (p=0.003) (Table 1).

Table 1: Fibrinogen, CRP and IL6 levels at baseline and 7 days after yellow fever vaccination.

	t=0	t=7	% change
Fibrinogen (g/l)	2.8 (2.2, 3.5)	3.1 (2.2, 3.9)	9.4 (-12.5, 44.1) *
CRP(mg/l)	0.8 (0.1, 4.1)	0.9 (<0.05, 4.8)	32.1 (-88.2, 672.2)
IL6 (pg/ml)	1.0 (0.5, 4.4)	0.8 (0.4, 4.1)	21.2 (-54.7, 447.7)

Data are median values (range), *p= 0.003

There were no significant correlations between the relative changes of fibrinogen, CRP and IL6, but there was a significant correlation between CRP levels and IL6 levels after vaccination (r=0.42, p=0.05). For all three inflammatory markers, the plasma levels at baseline correlated significantly with the levels of these proteins at 7 days after vaccination (r=0.67 p=0.001 for fibrinogen, r= 0.68 p=0.001 for CRP, r=0.57 p=0.006 for IL6).

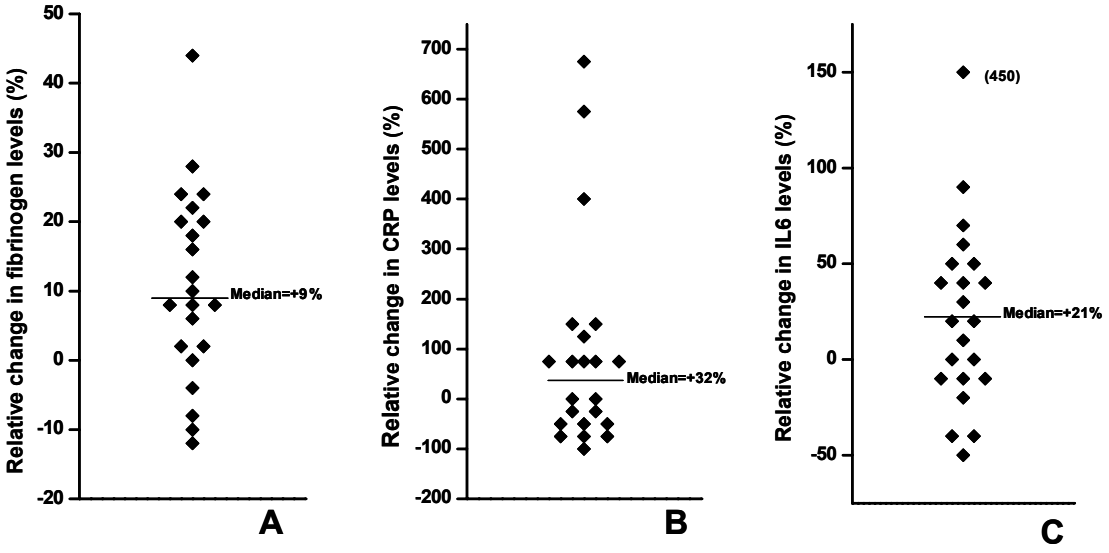

Figure 1: Relative change in fibrinogen, CRP and IL6 levels in response to yellow-fever vaccination.

Interindividual variation in the response by fibrinogen, CRP and IL6 plasma levels to yellow fever vaccination

Large interindividual variation was observed between the subjects with respect to the response by fibrinogen, CRP and IL6 levels to the yellow fever vaccination. For fibrinogen, the median increase in fibrinogen levels 7 days after vaccination was 9%, but varied between –13% and +44%. The median change in CRP levels was +32%, and showed a large variation between –88% and +672%. Finally, the median increase of IL6 levels was 21%, and varied between –55% and +448% (Table 1, Figure 1). All participants were healthy, and none of them had experienced illness during the study period, or reported other factors that could explain the large differences in response between the subjects.

Genetic variation influences the response to vaccination

We observed a significant association between the IL6 -174G/C genotype and the relative increase in CRP levels (Table 2, Figure 2). The CRP levels of subjects homozygous for the G allele of the -174G/C polymorphism showed a larger relative increase in response to the yellow fever vaccination than CRP levels of subjects carrying at least one C allele (p=0.032). For fibrinogen, there was a slightly stronger relative increase of fibrinogen levels in fibrinogen β -455A allele carriers compared to -455GG homozyotes, but the differences were not statistically significant (p=0.12). The genotype distributions of both polymorphisms were not different from those predicted by Hardy-Weinberg equilibrium.

Table 2: Association of fibrinogen β -455G/A and IL6 -174G/C genotype with the response of fibrinogen, CRP and IL6 levels to vaccination.

	N	Genotype	t=0	t=7	% change
Fibrinogen (g/l)	16	Fbg β -455GG	2.9 (2.3, 3.5)	3.1 (2.2, 3.9)	7.5 (-12.5, 28.9)
	6	Fbg β -455GA/AA	2.6 (2.2, 3.1)	3.0 (2.7, 3.7)	15.1 (5.9, 44.1)
CRP (mg/l)	7	IL6 -174GG	0.8 (0.1, 1.5)	1.3 (0.4, 4.8)	135.1 (-34.0, 672.2)*
	15	IL6 -174GC/CC	0.6 (0.1, 4.1)	0.5 (<0.05, 4.1)	-19.5 (-88.2, 563.6)*
IL6 (pg/ml)	6	IL6 -174GG	0.1 (0.5, 4.4)	1.1 (0.4, 4.1)	19.1 (-10.1, 86.3)
	16	IL6 -174GC/CC	0.1 (0.5, 2.4)	1.0 (0.6, 2.9)	23.3(-54.7, 447.7)

Data are median values (range), * p=0.032

***In vitro* whole-blood LPS stimulation assay**

After LPS stimulation, IL6 levels in the whole-blood samples strongly increased. The median IL6 level at baseline in the samples was 1.0 (0.5-4.4) (pg/ml), and after LPS treatment IL6 levels strongly increased to 607 (0-2536) pg/ml in response to 10 ng/ml LPS.

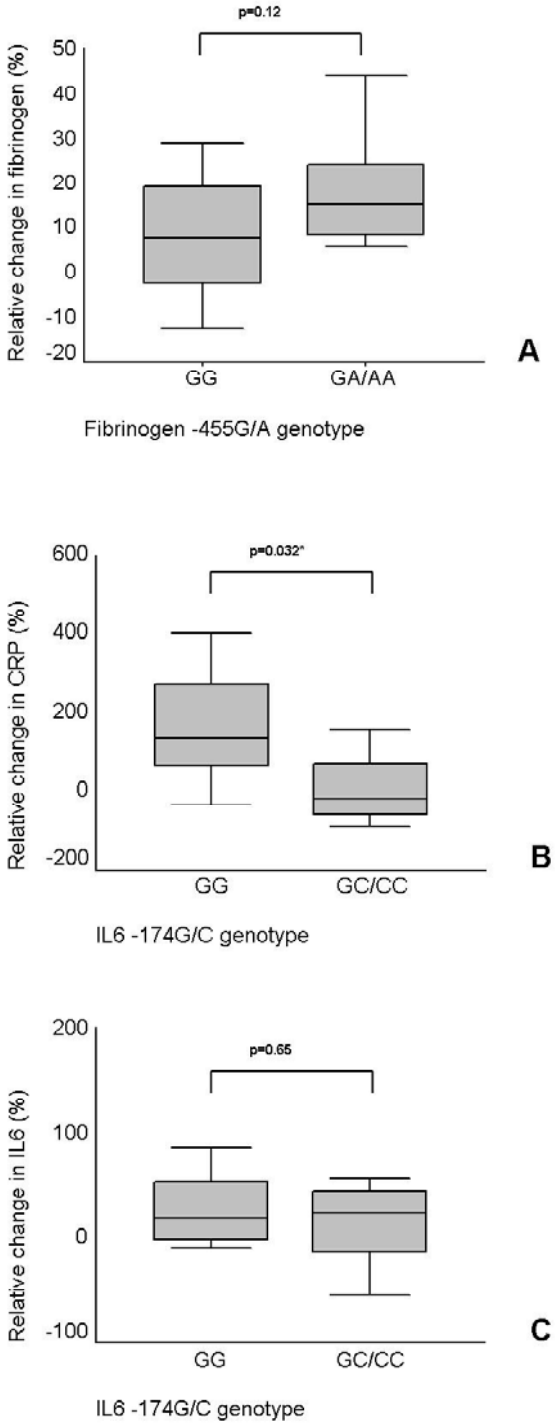

Figure 2: Relationships between genotype and inflammatory response.
Association between fibrinogen β -455G/A genotype and the response of fibrinogen levels to vaccination (Figure 2a), between IL6 -174G/C genotype and the response of CRP levels to vaccination (Figure 2b), and between IL6 -174G/C genotype and the response of IL6 levels to vaccination (Figure 2c).

When the *in vitro* increase in IL6 levels in response to LPS was compared with the *in vivo* increases of fibrinogen, CRP or IL6 plasma levels after yellow fever vaccination, no significant correlations were observed. Finally, there also was no relationship between IL6-174G/C genotype and the *in vitro* increase in IL6 levels in response to LPS.

DISCUSSION

This study shows that a large variation exists between individuals with respect to the response by fibrinogen, CRP and IL6 plasma levels to a mild, standardized inflammatory stimulus (yellow fever vaccination). Although the results may be limited by the small sample size of the population, this study indicates that genetic variation in the promoter regions of the inflammatory genes may contribute to the interindividual variation in response.

It was expected that large differences could exist between individuals with respect to their response to inflammatory triggers [1,21,22], and our study is the first to document the interindividual variation in the response by acute phase reactants to a mild and standardized inflammatory stimulus. In our study, in which we used yellow fever vaccination as a mild acute-phase trigger, there was much variation between individuals in the magnitude of change of fibrinogen, CRP and IL6 levels. Fibrinogen levels changed between -13% and +44%, and CRP levels between –88% and +672%, and IL6 levels between -55% and +448%.

In a previous pilot study, in which the plasma levels of fibrinogen, CRP and IL6 were measured daily, we showed that CRP and fibrinogen levels peak 7 days after vaccination [17]. This peak occurs ±3 days after the viraemia that results after the vaccination [15,16]. In the present study, which has the same experimental design as the pilot study, moderate changes in the plasma levels of fibrinogen, CRP and IL6 were observed, and a significant increase of the average fibrinogen level was detected.

We did observe significant relationships between baseline values and levels after vaccination for all three individual inflammatory markers. This suggests that subjects with higher basal levels are likely to have higher peak levels in response to inflammation. These findings are in line with another study, in which subjects with higher basal CRP and serum amyloid A (SAA) levels had higher peak levels of these proteins after angiography or PTCA [23]. Associations between plasma levels of fibrinogen, CRP and IL6 have been reported on several occasions [6,24], and in our study we observed significant correlations between CRP and fibrinogen levels at baseline, and between CRP and IL6 levels at day 7. Correlations with the other inflammatory markers might have been expected since IL6 is a primary inducer of both CRP and fibrinogen release, but the small size of our study population may account for this lack of association.

An alternative explanation for the lack of correlation of IL6 levels at day 7 with CRP and fibrinogen may be the fact that at day 7 the CRP and fibrinogen levels were at their peak values, whereas the IL6 peak may have been slightly earlier.

We observed that genetic variation contributes to the variation in response between individuals. CRP expression is strongly upregulated by IL6 [25], and the IL6 -174G/C promoter polymorphism has been associated with basal plasma CRP levels [26]. However, the results of the association studies published up until now are not consistent, as in some studies the -174G, and in other studies the -174C allele is associated with elevated basal CRP levels [26,27]. In functional studies, IL6-promoter-reporter gene constructs with the -174G allele responded more strongly to LPS or interleukin-1 than constructs with the -174C allele in one study [13], but in another study, this was less evident [14]. We observed that subjects homozygous for the G allele of the IL6 -174G/C polymorphism showed a significantly stronger response in CRP levels to yellow-fever vaccination, a mild acute phase trigger. For fibrinogen, we observed a trend towards a stronger response by fibrinogen levels to vaccination in -455A allele carriers, and a stronger response by fibrinogen levels in -455A allele carriers to inflammatory stimuli has been reported by others as well [10-12]. Perhaps remarkably, we did not observe a relationship between the -174G/C genotype and the response by IL6 levels. We attribute this to the fact that IL6 levels peak 5 days after vaccination, and we drew blood 7 days after vaccination. It is therefore probable that the peak in IL6 levels had already occurred at the time of the blood sampling, and that we were not able to detect an effect of the IL6 -174G/C genotype on the response by IL6 levels because of this. However, since we did observe a relationship between the IL6 -174G/C polymorphism and the response by CRP levels, we should expect that the -174G/C polymorphism had also affected the IL6 response.

A whole-blood *in vitro* LPS stimulation assay has been used previously to study the intensity of the response of an individual to potential inflammatory stimuli, and an association between the *in vitro* IL6 production after LPS-challenge and circulating CRP levels in patients with unstable angina has been reported [9]. However, in our LPS stimulation assays with blood samples from healthy subjects we did observe a strong response by IL6 expression to LPS treatment, but the increase in IL6 in response to LPS *in vitro* did not correlate with the response by fibrinogen, CRP and IL6 to vaccination *in vivo*. This indicates that this whole-blood stimulation assay is not a representative model for the *in vivo* response to yellow fever vaccination. This may be explained by the fact that LPS and yellow fever activate different receptors. The yellow fever virus has a single-stranded, positive polarity RNA genome of 10,862 nucleotides [28] and it has been shown recently that toll-like receptor 3 (TLR 3) is the receptor for viral RNA [29]. LPS however activates the TLR 4 [30], and therefore activation of different receptors by yellow fever and LPS could explain the lack of association between our *in vivo* and *in vitro* findings.

In conclusion, this study shows that there are large differences between individuals regarding the intensity of the response by CRP, fibrinogen and IL6 plasma levels to yellow fever vaccination, and that genetic variation may be one of the factors determining the responsiveness of an individual. This is a potentially relevant conclusion for several diseases that have an inflammatory component such as cardiovascular disease, but also for sepsis, malignancies, and infectious diseases. This study shows that individuals can be classified as hyper- or hypo-responders, which may be useful, as individuals hyper-responsive to inflammatory triggers may have a different disease risk profile. This work indicates that yellow fever vaccination provides an interesting tool for investigating the inflammatory response under standardized conditions, in larger studies. A relevant research aim in such future studies would be to study the biological mechanisms of acute-phase reactions. More understanding of the biological mechanisms of different acute-phase reactions would help in choosing the appropriate model system, for instance the *in vivo* yellow fever vaccination model or the *in vitro* whole-blood LPS stimulation assay, described in this study.

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Chapter 5

The plasminogen activator inhibitor-1 (PAI-1) promoter haplotype is related to PAI-1 plasma concentrations in lean individuals

Maartje Verschuur, Annemarie Jellema, Else M. Bladbjerg, Edith J.M. Feskens, Ronald P. Mensink, Lars Møller, Hans L. Vos and Moniek P.M. de Maat

Submitted

The plasminogen activator inhibitor-1 (PAI-1) promoter haplotype is related to PAI-1 plasma concentrations in lean individuals

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ABSTRACT

Elevated plasminogen activator inhibitor-1 (PAI-1) concentrations are associated with cardiovascular diseases. PAI-1 antigen levels are influenced by environmental factors such as body mass index (BMI), and by genetic factors. The -844A/G and the -675(4G/5G) polymorphisms are present in the PAI-1 promoter and they are in linkage disequilibrium. The 4G allele of the -675(4G/5G) variation has been associated with elevated PAI-1 concentrations and on some occasions with an increased risk of cardiovascular disease. The aim of our study was to investigate the effect of the PAI-1 promoter haplotype on PAI-1 concentrations and to determine the role of BMI. The association between the PAI-1 promoter haplotype and PAI-1 antigen levels was investigated in two populations, each including 600 healthy Caucasians. To assess the direct effect of the PAI-1 promoter haplotype on PAI-1 promoter activity, *in vitro* reporter gene assays were performed in HepG2 and BAEC cells. We observed significantly higher PAI-1 concentrations in A-4G homozygotes than in G-5G carriers, in the lowest BMI quartile. In these lean subjects, the PAI-1 concentrations of A-4G/G-5G heterozygotes were reduced to 60-75%, and the concentrations of G-5G homozygotes to 45-55%, compared to the PAI-1 concentrations of A-4G homozygotes. PAI-1 concentrations increased approximately 4-fold from the lowest to the highest BMI quartile. The *in vitro* reporter gene assays did not indicate a direct effect of the PAI-1 promoter haplotype on promoter activity. Our study suggests that the PAI-1 promoter haplotype and BMI affect PAI-1 concentrations and that BMI is a stronger determinant than PAI-1 promoter variation.

INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is a 50 kD plasma glycoprotein and a member of the serpin family. PAI-1 is produced in several tissues including liver, endothelium, adipose tissue, and vascular smooth muscle cells [1-3]. PAI-1 controls fibrinolysis by the inhibition of the tissue-type- and urokinase-type plasminogen activators, and increased PAI-1 plasma levels result in impaired fibrinolytic capacity [4]. Elevated PAI-1 levels have been associated with an increased risk of cardiovascular disease in epidemiological studies [5]. Moreover, elevated PAI-1 levels are associated with an elevated body mass index (BMI), hypertension, hyperinsulinaemia, lipid disorders and increased cytokine levels, together referred to as the metabolic syndrome [6]. This shows that PAI-1 levels, cardiovascular disease and several traditional risk factors (e.g. BMI, hyperinsulinaemia and hypertension), are tightly interrelated.

PAI-1 levels are also partly determined by genetic variables, and the heritability of PAI-1 levels in the normal population is estimated at 50-60% [7]. Two common polymorphisms have been described in the promoter region of the PAI-1 gene: the -844A/G (*Xho*I) polymorphism, and the -675(4G/5G) polymorphism. These polymorphisms are in strong, but not complete, positive linkage disequilibrium in the Caucasian population [8]. The 4G allele of the -675(4G/5G) variation is associated with elevated PAI-1 levels, and an association of the 4G allele with an increased risk of coronary artery disease and myocardial infarction has been reported on some occasions [9-12]. Functional studies have indicated that the -675(4G/5G) polymorphism may directly affect PAI-1 gene transcription, especially in response to factors such as cytokines, lipoproteins and hormones [13,14]. In addition, some association studies suggest that the relationship between PAI-1 levels and metabolic factors such as BMI and plasma lipids, may be influenced by the -675(4G/5G) polymorphism [9,15,16]. However, this interaction was not observed in other epidemiological studies [3,8]. In addition, it has been suggested that the -844A/G polymorphism may also be relevant. The -844A allele has been associated with an increased risk of venous thrombosis in carriers of the Factor V Leiden mutation, and it has been shown *in vitro* that the -844A/G variation can affect the binding of nuclear proteins to the PAI-1 promoter [17,18]. However, as the two variations are in linkage disequilibrium, the relevance of the individual -844A/G and -675(4G/5G) variations is still under discussion. Therefore, a haplotype-based approach was used in this study.

The aim of this study was to determine the contribution of the PAI-1 promoter haplotype to PAI-1 plasma levels, and the modification of this relationship by BMI. In addition, we performed *in vitro* functional assays to determine the direct effect of PAI-1 promoter haplotype on PAI-1 promoter activity. Besides the -675(4G/5G) variation, these functional assays also included the less-well characterized -844A/G variation.

METHODS

Study populations

Analyses were performed separately in two populations which have been described in more detail elsewhere [19,20]. Briefly, the first population was originally designed to investigate the *Gly972Arg* polymorphism in the insulin receptor-substrate-1 (IRS-1) gene. Therefore, carriers of the rare *972Arg* allele were oversampled from two cohorts of healthy Caucasians: the Maastricht area of the Cardiovascular Disease Risk Factor Monitoring Project and the Dutch Monitoring Project on Risk Factors for Chronic Diseases (MORGEN) [21], and this combined population will be referred to as the MORGEN sample [20]. DNA and plasma samples were available from 564 subjects, and PAI-1 promoter haplotype combinations and PAI-1 antigen (ag) levels could be obtained from 561 subjects. As the IRS-1 gene and the PAI-1 gene are located on different chromosomes, no effect of oversampling of the IRS-1 *972Arg* allele on the PAI-1 haplotype frequencies was expected.

The second population is the Glostrup study, a cohort of healthy individuals (n=1198) born in 1936 and living in the area served by the Glostrup Hospital in Copenhagen, Denmark, which has been followed since 1976 [19,22]. In 1996, the cohort was re-examined with a participation rate of 65% (695 subjects). DNA and plasma samples were available from 664 subjects, and PAI-1 promoter haplotype combinations and PAI-1 antigen levels could be obtained from 631 subjects.

For both the MORGEN sample and the Glostrup study, the subjects provided informed consent and the study protocols were approved by the local medical ethical committees.

Blood sampling, DNA extraction, biochemical procedures

Collection of samples and biochemical procedures in the MORGEN sample and the Glostrup study are described in more detail elsewhere [19,20]. Briefly, fasting blood samples were drawn into tubes containing citrate in the MORGEN sample, and in Stabylite tubes (Biopool) in the Glostrup study. PAI-1 antigen concentrations in plasma were measured using EIA (MORGEN: Elitest PAI-1, Hyphen Biomed and Glostrup: Imulyse PAI-1:Ag, Biopool). DNA was isolated from buffy coats. Triglyceride, total cholesterol, HDL cholesterol, pro-insulin and glucose plasma levels were measured in the MORGEN sample as described previously [20], and enzymatic methods were used to assess the serum concentrations of triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol in the Glostrup study (Roche).

Haplotyping of the PAI-1 promoter

To determine the PAI-1 promoter haplotypes in the MORGEN sample, a PCR was designed with primers specific to either the -675(4G) or the -675(5G) allele (Figure 1). In this reaction, 0.4 μM forward primer (5'-GCT TGA ATC ATC CCG AAA CCA TCC C-3'), 0.4 μM reverse allele specific primer (4G allele: 5'-TAC ACG GCT GAC TCC CCA-3', 5G allele: 5'-TAC ACG GCT GAC TCC CCC-3'), and 0.04 μM reverse control primer (5'-TGC AGC CAG CCA CGT GAT TGT CTA-3'), were used at an annealing temperature of 66°C. Separate reactions were carried out with the 4G- and 5G-specific primers for each DNA sample and the control primer was included to avoid false negatives. PCR products were digested with *Xho*I (Invitrogen) and the restriction patterns on agarose gel revealed the PAI-1 promoter haplotype.

As the G-4G haplotype occurred at a frequency of only 0.4% in the MORGEN sample, it was investigated whether two single genotypes can be used reliably to construct the haplotype. In the MORGEN sample, an Expectation-Maximization algorithm was used to estimate the haplotypes based on the frequencies of the individual genotypes. It was calculated that the chance of mistyping an individual heterozygous at both polymorphic sites was 0.1%. Because of this low chance of mistyping, for the Glostrup study haplotype combinations were constructed based on the individual 844A/G and -675(4G/5G) genotypes, and subjects heterozygous at both polymorphic sites were assigned the haplotype combination A-4G/G-5G. Previously described methods to determine the -675(4G/5G) and the -844A/G polymorphisms individually were used [8,19].

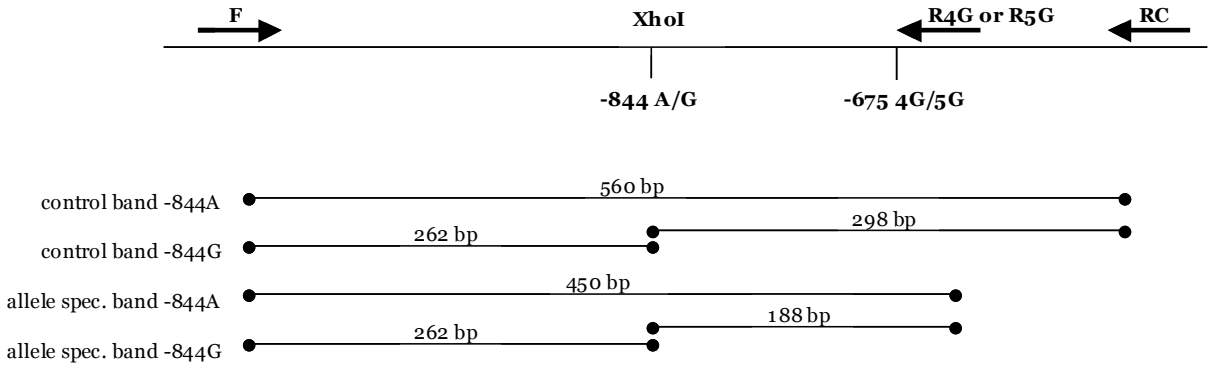

Figure 1: The promoter region of the PAI-1 gene. The -844A/G and the -675(4G/5G) variations and the locations of the haplotype-specific PCR primers are shown. The bottom part shows the fragments formed after digestion of the PCR products with *Xho*I.

Functional PAI-1 promoter assays

CELLS AND REAGENTS

HepG2 hepatoma cells were obtained from American Type Culture Collection, and maintained in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Bio Whittaker Europe) and 10% foetal bovine serum (FBS, Invitrogen). Bovine aorta endothelial cells (BAECs) were isolated by scraping the inner surface of calf aortas with a scalpel, and maintained in HepG2 culture medium supplemented with 5 IE/ml heparin (Leo Pharma BV) in plates or flasks coated with 1% gelatin.

REPORTER GENE CONSTRUCTS, TRANSFECTION AND LUCIFERASE ASSAY

2.0 kb of the promoter region of the PAI-1 gene (-1979 to +23, relative to the transcription start site) was amplified by PCR (forward primer: 5'-TCT AAC GCG TCA TCC ACA ACA TCC AGA CCA-3', reverse primer: 5'-TAC TAG ATC TCC CTG CAG CCA AAC ACA G-3'). PCR products were digested with *Mlu*I and *Bgl*II and cloned into pGL3-basic (Promega), resulting in PAI-1 promoter-reporter gene constructs with the A-4G haplotype or the G-5G haplotype. By the exchange of the *MluI-Bsu36I* restriction fragment between these haplotype constructs, constructs with the G-4G and the A-5G haplotypes were derived. Constructs were sequenced and no additional polymorphisms were detected.

HepG2 cells were plated in 24-well plates. After an overnight incubation, medium was replaced with DMEM supplemented with penicillin and streptomycin and 0.1% human serum albumin (HSA, Cealb®). After 2h, cells were transfected using FuGene 6 (Roche). 25 ng pGL3 construct, 250 ng carrier DNA (herring sperm, Invitrogen) and 0.25 ng pRL-SV40 (Renilla luciferase expression construct, Promega) were used per well. 24h after transfection, medium was replaced and cells were cultured for an additional 24h. BAEC cells were plated in 24-wells plates. After an overnight incubation, cells were transfected by calcium phosphate precipitation [23]. 500 ng pGL3 construct and 50 ng pRL-SV40 construct were used per well. Transfection medium was removed 6h after transfection, cells were washed and DMEM supplemented with penicillin, streptomycin and 0.25% HSA was added. For each construct two independent DNA preparations were transfected twice in triplicate. After culturing the cells for 48h following transfection, cells were washed, lysed and Firefly luciferase and Renilla luciferase activities were measured in lysates using the Dual-Luciferase® Reporter Assay System (Promega).

STATISTICAL ANALYSIS

Variables with a skewed distribution (PAI-1 antigen, triglycerides, glucose and pro-insulin) were logarithmically (ln) transformed. PAI-1 antigen data are presented as geometric means (±SDs). Pearson correlation coefficients were

calculated between PAI-1 concentrations and the metabolic variables. In both populations BMI had the highest correlation coefficient with PAI-1 concentrations, therefore the population was divided into quartiles according to BMI. Comparisons were performed by one-way ANOVA. If applicable, Least-significant difference (LSD) post hoc tests were performed. $P<0.05$ was considered statistically significant. For the sake of clarity, PAI-1 antigen levels are expressed in relation to the geometric mean of the populations in the figures.

In the functional assays, firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity as internal standard, and expressed as a percentage of normalized luciferase activity of haplotype A-4G. Normalized expression levels were compared by ANOVA testing.

For all the analyses described above, SPSS 11.5 for Windows was used. An Expectation-Maximization algorithm, incorporated in Arlequin, was used to estimate the PAI-1 promoter haplotype combinations in the Glostrup population [24].

RESULTS

PAI-1 promoter haplotypes and PAI-1 antigen levels

BASELINE CHARACTERISTICS

The baseline characteristics of the MORGEN and the Glostrup study populations are shown in Table 1.

Table 1: Baseline characteristics of the MORGEN and Glostrup populations

	MORGEN N=564		Glostrup N=664	
	Mean (±SD)	Range	Mean (±SD)	Range
Age (years)	54.2 (±10.0)	28.0-73.0	60 ‡	
Men	45.30%		46.80%	
BMI (kg/m²)	26.6 (±5.2)	17.8-41.0	26.5 (±3.9)	17.3-46.9
PAI-1 antigen (ng/ml) *	78.7 (±31.6)	7.3-729.8	10.1 (±5.1)	1.0-85.8

‡ All subjects in the Glostrup study were of the same age, * because of the skewed distribution, the geometric means (±SD) of PAI-1 antigen levels (MORGEN: Elitest, Glostrup: Imulyse) are shown.

Mean PAI-1 antigen level in the MORGEN sample was 78.7 ng/ml (Elitest PAI-1, Hyphen Biomed), and in the Glostrup study 10.1 ng/ml (Imulyse PAI-1:Ag, Biopool). The difference in absolute PAI-1 concentrations between the

populations can be attributed to the different assays used in the studies. In both study populations, the range of the PAI-1 levels was over two orders of magnitude, and the PAI-1 concentrations of men were significantly higher than those of women (in both cases $p<0.01$).

DISTRIBUTION OF PAI-1 PROMOTER HAPLOTYPES AND ASSOCIATION WITH PAI-1 ANTIGEN LEVELS

The frequencies of the four PAI-1 promoter haplotypes in both populations are shown in Table 2. The -675(4G) allele was almost completely associated with the -844A allele, whereas the -675(5G) allele occurred with both -844A and G alleles, although the combination with the -844G allele was more frequent.

As BMI was the strongest metabolic contributor to PAI-1 antigen levels (Table 3), the relationship between the PAI-1 promoter haplotype and PAI-1 concentrations were investigated in the two populations as a whole, and by BMI quartiles in the MORGEN sample and the Glostrup study separately (Figures 2 and 3).

Table 2: PAI-1 promoter haplotype frequencies in the MORGEN and Glostrup populations

Haplotype		MORGEN	Glostrup
Full name	**Abbrev.**	**N**	**N**
-844A/-675(4G)	**A-4G**	595 (52.7%)	705 (53.1%)
-844G/-675(4G)	**G-4G**	4 (0.4%)	5 (0.4%)
-844A/-675(5G)	**A-5G**	67 (5.9%)	68 (5.1%)
-844G/-675(5G)	**G-5G**	462 (41.0%)	550 (41.4%)
Total		1128	1328

Table 3: Pearson correlation coefficients between PAI-1 ag levels, metabolic variables and age.

	MORGEN N=564		Glostrup N=664	
	R	p	R	p
Age	0.34	<0.001	ND *	
BMI	0.63	<0.001	0.44	<0.001
pro insulin ‡	0.5	<0.001	ND	
Glucose ‡	0.48	<0.001	0.29	<0.001
TG ‡	0.48	<0.001	0.37	<0.001
VLDL cholesterol	ND		0.33	<0.001
HDL cholesterol	-0.44	<0.001	-0.32	<0.001
LDL cholesterol	ND		0.14	<0.001
total cholesterol	0.13	<0.001	0.09	<0.05

PAI-1 antigen levels and ‡ levels were ln-transformed prior to analysis, * All subjects in the Glostrup study were of the same age, therefore no correlation between age and PAI-1 antigen levels could be determined (ND= not determined).

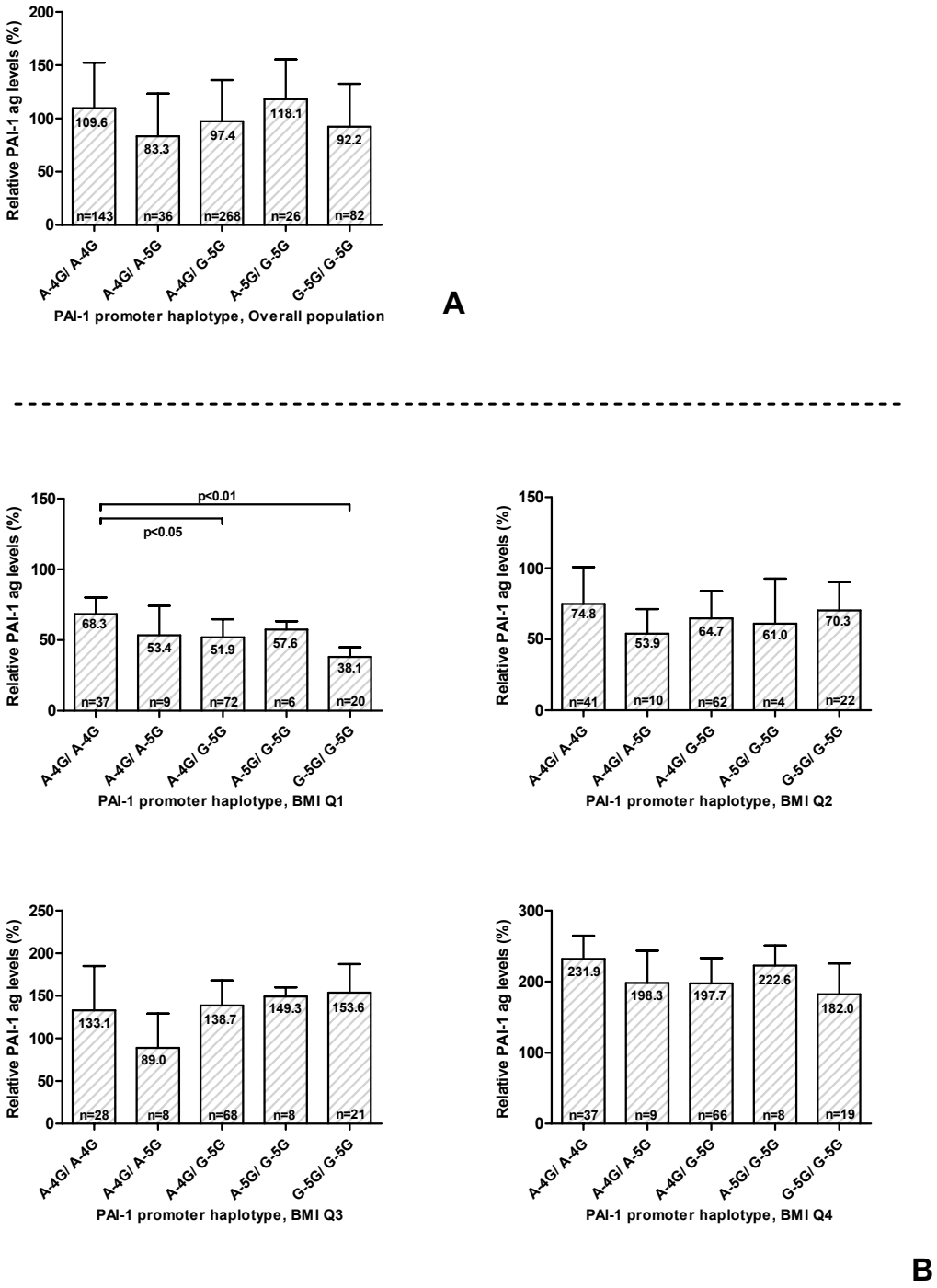

Figure 2: PAI-1 antigen levels of the haplotype combinations in the overall MORGEN sample (Figure 2A) and in BMI Quartiles of the MORGEN sample (Figure 2B). Bars represent the geometric means (+SD) of the PAI-1 antigen levels expressed as a percentage of the geometric mean of the overall population, for each haplotype combination separately. Relative geometric means and number of subjects are indicated. Rare haplotype combinations are not shown, their relative PAI-1 antigen levels are: Quartile 2: (5A/5A: 148.9(n=1)), Quartile 3: (4A/4G: 92.3(n=1)), (4G/5G: 163.1(±55.7)(n=2)), Quartile 4: (4G/4G: 184.1(n=1)), (5A/5A: 227.7(n=1)).

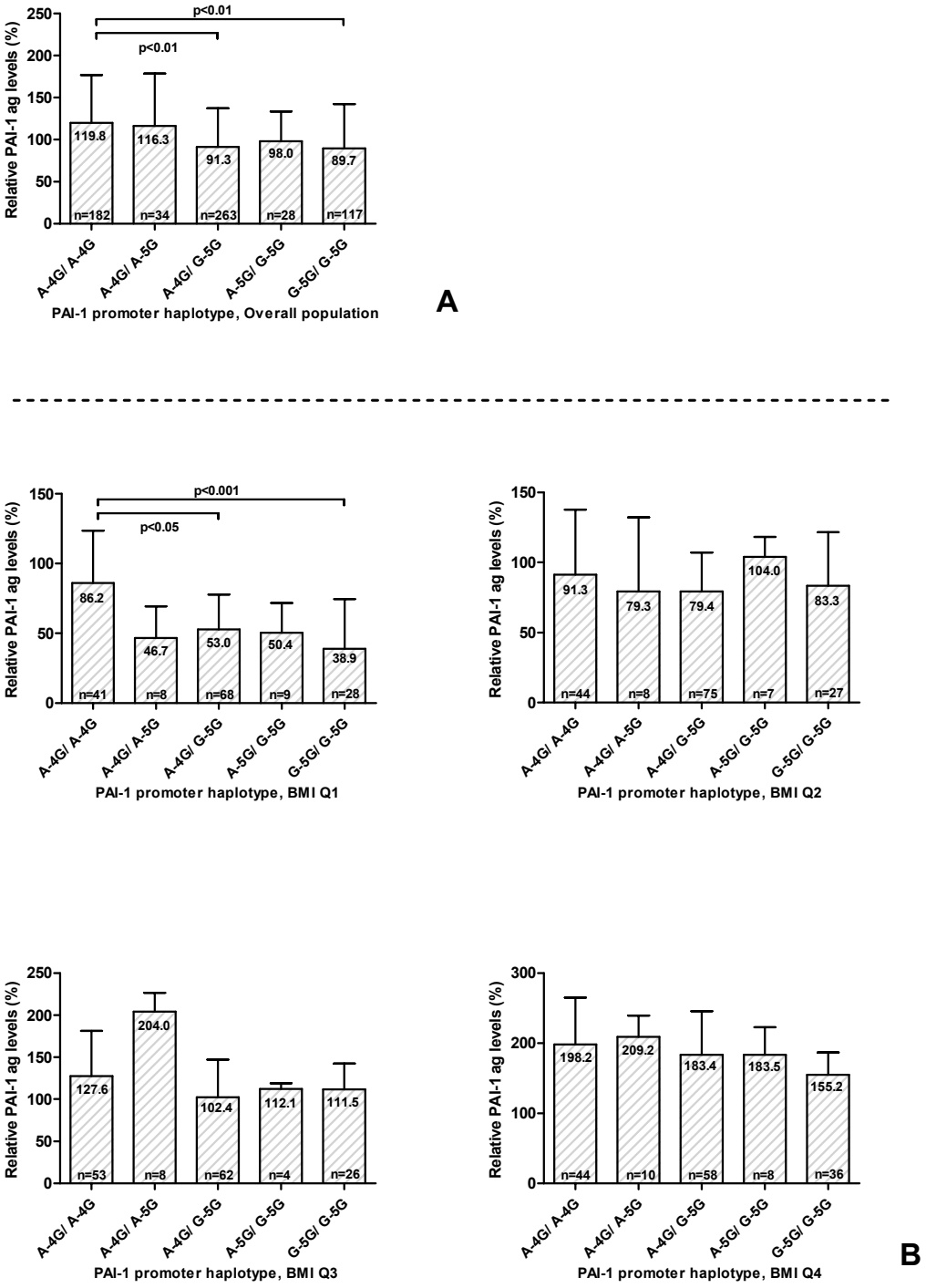

Figure 3: PAI-1 antigen levels of the haplotype combinations in the overall Glostrup population (Figure 3A) and in BMI Quartiles of the Glostrup population (Figure 3B). Bars represent the geometric means (+SD) of the PAI-1 antigen levels expressed as a percentage of the geometric mean of the overall population, for each haplotype combination separately. Relative geometric means and number of subjects are indicated. Rare haplotype combinations are not shown, their relative PAI-1 antigen levels are: Quartile 1: (4A/4G: 28.6(±6.5)(n=2)), Quartile 3: (4A/4G: 82.3(±18.1)(n=3)), (5A/5A: 175.7(n=1)), Quartile 4: (5A/5A: 529.1(n=1)).

In the MORGEN sample overall, there were no significant differences in PAI-1 concentrations between the haplotype combinations (Figure 2A). However, in the lowest BMI quartile of the MORGEN sample (BMI< 22.0 kg/m²), PAI-1 antigen levels differed significantly between the promoter haplotype combinations ($p<0.05$). The PAI-1 concentrations of A-4G homozygotes were significantly higher than those of A-4G/G-5G heterozygotes ($p<0.05$), and than those of G-5G homozygotes ($p<0.01$). The geometric mean PAI-1 concentrations of A-4G/G-5G heterozygotes and G-5G homozygotes were respectively 76% and 56% of the geometric mean PAI-1 levels of A-4G homozygotes (Figure 2B).

In the Glostrup population overall, significant differences in PAI-1 concentration between the haplotype combinations were observed ($p<0.05$). The PAI-1 antigen levels of A-4G homozygotes were significantly higher than those of A-4G/G-5G heterozygotes ($p<0.01$), and than those of G-5G homozygotes ($p<0.001$) (Figure 3A). When the population was divided into BMI quartiles however, these differences were only present in the lowest BMI quartile (BMI< 23.8 kg/m²) ($p<0.05$), similarly to the observations in the MORGEN sample.The PAI-1 levels of A-4G homozygotes were significantly higher than those of A-4G/G-5G heterozygotes ($p<0.05$), and than those of G-5G homozygotes ($p<0.001$). In the lowest BMI quartile, the geometric mean PAI-1 antigen level of A-4G/G-5G heterozygotes and G-5G homozygotes were respectively 61% and 45% of the geometric mean PAI-1 levels of A-4G homozygotes (Figure 3B).

In both the MORGEN sample and the Glostrup study, no significant differences in PAI-1 antigen level between the haplotype groups were detected in the 2nd, 3rd and 4th BMI quartiles (Figures 2 and 3).

Table 4: PAI-1 promoter activity in hepatoma and endothelial cells

Haplotype		PAI-1 promoter activity	
Full name	**Abbrev.**	**HepG2**	**BAEC**
-844A/-675(4G)	**A-4G**	100 (±10.3)	100 (±21.9)
-844G/-675(4G)	**G-4G**	95 (±9.0)	104 (±20.7)
-844A/-675(5G)	**A-5G**	88 (±7.6)	93 (±50.1)
-844G/-675(5G)	**G-5G**	91 (±16.7)	123 (±27.3)
Anova		p=0.08	p=0.44

Normalized luciferase activities are expressed in relation to the activity of the A-4G haplotype construct. Means (±SD) of 12 transfections obtained in four independent experiments are shown.

Functional PAI-1 promoter assays

To investigate whether PAI-1 promoter variation directly affects PAI-1 promoter activity, reporter-gene assays were performed, with constructs representing the

four different PAI-1 promoter haplotypes. No significant differences between the basal promoter activity of the four PAI-1 promoter haplotypes were observed in hepatoma (HepG2) or endothelial (BAEC) cells (Table 4), although the lowest promoter activity was seen for the G-5G and A-5G promoter haplotypes.

DISCUSSION

In this study we observed associations between PAI-1 promoter haplotypes and PAI-1 concentrations, which were most pronounced in subjects in the lowest BMI quartile. *In vitro* reporter gene assays showed no clear indications of a direct effect of the PAI-1 promoter variation on PAI-1 promoter activity.

To study the relationship between PAI-1 promoter haplotype and PAI-1 antigen levels, we determined the PAI-1 promoter haplotypes in two different populations of healthy individuals, and in both populations the frequencies of the haplotypes were similar. The A-4G haplotype had a frequency of approximately 53%, the G-4G haplotype a frequency of 0.4%, the A-5G haplotype a frequency of approximately 6%, and the G-5G haplotype a frequency of approximately 41%. This is the first time that the frequencies of the PAI-1 promoter haplotypes have been reported. The haplotype frequencies are in accordance with the reported allele frequencies of the individual -844A/G and -675(4G/5G) variations [8,17,25].

In both populations, homozygotes for the A-4G haplotype had higher PAI-1 concentrations than A-4G/G-5G heterozygotes and G-5G homozygotes. In the MORGEN sample, this association was only significant in the lowest BMI quartile (BMI<22.0). In the Glostrup study the association was also present in the whole population, but when the population was divided into BMI quartiles, the association between PAI-1 plasma levels and PAI-1 promoter haplotypes was only significant in the lowest quartile (BMI<23.8). These results indicate that the association between PAI-1 promoter variation and PAI-1 levels is most prominent in lean individuals. To our knowledge, this is the first report on the relationship between PAI-1 promoter variation and PAI-1 concentrations in this subgroup. As expected, PAI-1 concentrations did tightly correlate with BMI in our populations, and mean PAI-1 antigen levels increased approximately four-fold from the lowest up to the highest BMI quartile. The confinement of the association between PAI-1 promoter haplotype and PAI-1 concentrations to the lowest BMI quartile suggests that that the effect of genetic variation of the PAI-1 promoter on PAI-1 concentrations is lost to the background of the (stronger) effect of BMI on these levels in the 2nd, 3rd and 4th BMI quartiles. More generally, metabolic factors such as BMI, are more important determinants of PAI-1 concentrations than genetic variation of the PAI-1 promoter, and this conclusion is supported by others [26]. This implies that we did not find

indications of a synergistic interaction between PAI-1 promoter genotype and PAI-1 antigen levels, which was suggested by some studies [9,16], but not by others [8,26].

The differences in PAI-1 levels between the PAI-1 promoter haplotype groups are fairly large (approximately 50% between A-4G homozygotes and G-5G homozygotes in the lowest BMI quartile). Therefore it is interesting to hypothesize how this compatible is with previous reports on the relationships between PAI-1 promoter variation, PAI-1 concentrations and the risk of cardiovascular disease. The reported relationships between PAI-1 promoter variation and PAI-1 antigen levels, and between PAI-1 antigen levels and the risk of cardiovascular disease are consistent, but the relationship between PAI-1 promoter variation and the risk of cardiovascular disease is debatable [27]. Our study indicates that the influence of genetic variation of the PAI-1 promoter on PAI-1 concentrations is reasonably strong mainly in lean individuals, i.e. individuals with a favourable status with respect to the important cardiovascular risk factor BMI. This could explain the reported weak (or absent) relationship between variation of the PAI-1 gene and the risk of cardiovascular disease [12,25].

It would be interesting to evaluate the individual effects of the -675(4G/5G) and the -844A/G polymorphisms. However, due to the low frequency of the G-4G and A-5G haplotypes in our populations, we could not discriminate between the effects of the -675(4G/5G) and -844A/G polymorphisms *in vivo*. The *in vitro* reporter gene assays with the four different PAI-1 promoter haplotypes provided an alternative approach to answering this question. In our *in vitro* studies, we did not find any clear indications of a direct effect of the PAI-1 promoter variation on basal PAI-1 promoter activity. These observations are in line with the findings of others [13,28], but in contrast to one positive report [10]. In this latter study constructs with two tandem copies of a 30 bp DNA segment containing either the 4G or the 5G allele upstream from a minimal promoter were used. In all the studies including ours which did not find any effect of promoter variation on basal PAI-1 promoter activity, a larger part of the proximal PAI-1 promoter with the -675(4G/5G) polymorphism in its natural context was used. This different design of the constructs which is used, is a plausible explanation for the inconsistency in the reported results. With the present work we confirm previous findings that -675(4G/5G) does not affect basal PAI-1 promoter activity in HepG2 and endothelial cells, and we show that also the -844A/G variation does not directly affect basal PAI-1 promoter activity in these cell types. However, we need to make the proviso that the cell culture conditions may not accurately represent the situation in the human body, and therefore we may have to be careful with the interpretation of these results.

We observed an association between PAI-1 promoter variation and PAI-1 plasma levels *in vivo*, in line with previous reports. Our *in vitro* observation that the PAI-1 promoter polymorphisms do not directly affect basal PAI-1

promoter activity also is consistent with the findings of others. This apparent discrepancy between the *in vivo* and *in vitro* results could be explained by a functional PAI-1 polymorphism located outside the promoter region of the gene, which was not included in our functional assays, but is responsible for the effects observed *in vivo*. For example, this additional polymorphism could be located in the 3' region of the gene and influence mRNA stability, or it could be located in the introns of the gene in the vicinity of splicing sites.
Another explanation for the lack of effect of genetic variation on PAI-1 promoter activity in HepG2 and BAEC cells could be that *in vivo* tissues other than liver and endothelium are responsible for the genotype-dependent PAI-1 expression. This is a relevant issue, as the transcriptional regulation of many genes, including the PAI-1 gene, can be highly dependent on the cell type [29]. Because of the relationship between BMI and PAI-1 plasma levels, there is much interest in the role of adipose tissue in the production of PAI-1. However, also for adipose tissue, no effect of the -675(4G/5G) polymorphism on the endogenous PAI-1 production could be detected [3], and therefore it is not yet clear which tissue is responsible for the genotype-dependent PAI-1 production *in vivo*.

In this study we observed a great difference between the average PAI-1 concentrations in the two populations, which can be attributed to the different PAI-1 immunoassays used. The PAI-1 antigen levels in the MORGEN sample (measured with Elitest) are in accordance with reported levels obtained with this assay [30], and this is also the case for the PAI-1 antigen levels in the Glostrup study (measured with Imulyse) [11,15]. The two assays differ in their specificity for the different PAI-1 forms, as Elitest recognizes all PAI-1 forms (active, in complex, inactive) equally well, whereas Imulyse is relatively insensitive to inactive PAI-1 [31].

In conclusion, in this study we observed an association between the PAI-1 promoter haplotype and PAI-1 antigen levels, which was most prominent in lean individuals. Our results indicate that both genetic variation and BMI influence PAI-1 levels, but that BMI is a more important determinant of PAI-1 concentrations. *In vitro* we did not observe any direct effect of the PAI-1 promoter haplotype on basal PAI-1 promoter activity in hepatoma and endothelial cells.

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Chapter 6

Genetic and environmental determinants of the plasminogen activator inhibitor-1 (PAI-1) concentration and their interactions

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ABSTRACT

Plasminogen activator inhibitor type 1 (PAI-1) is a main inhibitor of fibrinolysis and increased plasma PAI-1 levels are associated with an increased risk of cardiovascular disease. PAI-1 concentrations are regulated by environmental and genetic factors, and are associated with plasma levels of inflammatory factors and with features of the metabolic syndrome. The aim of this study was to describe the relationships between environmental and genetic factors and PAI-1 antigen levels, with a focus on the metabolic syndrome and inflammation. PAI-1 antigen levels, metabolic and inflammatory parameters, and the PAI-1 -675(4G/5G), insulin receptor substrate-1 (IRS-1) *Gly*972*Arg*, tumor necrosis factor-α (TNF-α -308G/A), and interleukin-6 (IL6) -174G/C genotypes were determined in a population of 600 healthy Caucasian subjects. Strong correlations were present between PAI-1 antigen levels and the plasma levels of several metabolic factors, and C-reactive protein. In addition, we observed that high body mass index (BMI) and low physical activity were associated with elevated PAI-1 concentrations (in both cases $p<0.001$). Concerning the genetic factors, a significant relationship with PAI-1 antigen levels was observed for the PAI-1 -675(4G/5G) polymorphism after adjustment for age, sex, the metabolic syndrome and environmental factors ($p=0.011$), and for the IRS-1 *Gly*972*Arg* polymorphism ($p=0.008$). In addition, our data indicate an interaction between the TNF-α -308G/A polymorphism and physical activity on plasma PAI-1 concentrations (p for interaction = 0.05), with higher PAI-1 concentrations in physically inactive subjects homozygous for the TNF-α-308A allele. This study demonstrates the effects of physical activity, BMI and the PAI-1 -675(4G/5G) and the IRS-1 Gly972Arg polymorphisms on plasma PAI-1 levels. We also obtained indications for an interaction between the TNF-α -308G/A polymorphism and physical activity. Our study suggests that these factors affect PAI-1 concentrations independent of the metabolic syndrome.

INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is a 50 kD plasma glycoprotein and a member of the serpin family. PAI-1 is produced in several tissues, including liver, endothelium, adipose tissue, and vascular smooth muscle cells [1-5]. PAI-1 controls fibrinolysis by inhibition of both tissue-type- and urokinase-type plasminogen activators. Increased PAI-1 plasma levels result in impaired fibrinolytic capacity [6], and elevated PAI-1 plasma levels are associated with an increased risk of cardiovascular disease [7,8].

The PAI-1 plasma concentration is determined by metabolic, environmental and genetic factors. The main determinant of the plasma PAI-1 concentration is the metabolic syndrome [9]. Elevated PAI-1 concentration in the metabolic syndrome may be the result of enhanced PAI-1 production in response to substances such as insulin, glucose, fatty acids or lipoproteins [10-14]. There is a strong relationship between body mass index (BMI), which is a major feature of the metabolic syndrome, and PAI-1 plasma levels. In addition, it has been demonstrated that weight reduction is effective in lowering PAI-1 levels [15]. Adipose tissue is an important source of PAI-1 in the circulation [16,17], which might explain the relationship between BMI and the PAI-1 plasma concentration. Insulin resistance also is an important feature of the metabolic syndrome, and several studies have indicated a relationship between insulin resistance and PAI-1 levels [9,18]. In addition, it has been reported that genetic variation of the insulin receptor substrate-1 (IRS-1) gene, which plays a key role in the cellular response to insulin, was associated with PAI-1 plasma levels [19].

Environmental factors such as smoking, physical activity and alcohol consumption also influence the PAI-1 concentration [20,21]. However, it is unclear if these variables influence PAI-1 concentrations independently of the insulin resistance syndrome. PAI-1 expression is also controlled by inflammatory factors, and tumor necrosis factor-α (TNF-α) is a key player in both inflammation and the obesity-linked elevation of plasma PAI-1 [22,23].

The heritability for the PAI-1 concentration is estimated at 50-60% [24,25]. Several polymorphisms in the PAI-1 gene have been described. The -675(4G/5G) single base pair insertion-deletion polymorphism has been studied on many occasions, and several studies have indicated that the 4G allele of this polymorphism is associated with elevated PAI-1 plasma levels [26-29]. It can also be anticipated that polymorphisms in genes upstream in the regulatory pathway of PAI-1 production may affect PAI-1 concentrations. Polymorphisms in genes regulating the inflammatory response (e.g. TNF-α or interleukin-6 (IL6)), or in genes important in the metabolic syndrome (IRS-1), could therefore be particularly interesting.

Therefore, the aim of this study was to describe the impact of environmental and genetic factors on the PAI-1 concentration, in a representative sample of the Dutch population.

METHODS

Subjects

Briefly, the study population of healthy individuals was selected from participants of the Maastricht area of the Cardiovascular Disease Risk Factor Monitoring Project and the Dutch Monitoring Project on Risk Factors for Chronic Diseases (MORGEN) [30,31]. All participants of these two studies were re-approached in 1998 with a self-administered questionnaire with questions on general health. This study was designed to study the (relatively rare) IRS-1 *Gly*972*Arg* polymorphism. Therefore, the IRS-1 *Gly*972*Arg* polymorphism was determined in all subjects and a subset of carriers of the rare *Arg* allele (n=519), and a random sample of the wild-type subjects (n=497) were selected, and invited for a new physical examination. Sixty percent of the invitees participated, and subjects with diagnosed diabetes were excluded [32]. All individuals for which the PAI-1 antigen level and the PAI-1 -675(4G/5G) genotype were available, were included in the analysis (n=561). Participants filled in an informed consent form, and the protocol was approved by the Medical Ethical Committee of TNO Prevention and Health, Leiden, the Netherlands.

Clinical measurements

After an overnight fast, measurements for the determination of BMI and waist-to-hip ratio (WHR) were obtained. Blood was drawn between 8.00 and 10.30 in the morning, into tubes containing K_3EDTA and in tubes containing sodium citrate. Subjects also handed in a general health questionnaire, which included questions about smoking behavior, alcohol consumption and physical activity. Subjects were classified as smokers or non-smokers. Alcohol consumption was categorized as follows: no alcohol, 0-1 glasses a day, or more than 1 glass a day. Bouts of exercise, biking, gardening, etc. were included to assess physical activity if they lasted 30 minutes per day or more. Subjects who reported such activities on three or more days a week were considered to be physically active, others were classified as non-active.

Laboratory measurements

PAI-1 antigen concentrations were determined using an enzyme immunoassay (EIA, Elitest PAI-1, Hyphen Biomed, France) in citrated plasma. In EDTA-plasma, triglyceride, HDL cholesterol, insulin and glucose levels were measured as described previously [32], and C-reactive protein (CRP) was determined with an in-house highly sensitive EIA, using polyclonal antibodies to human CRP as catching and tagging antibodies (DAKO Diagnostics) [33]. Genomic DNA was extracted from white blood cells, and the PAI-1 -675(4G/5G) polymorphism was determined by an allele-specific PCR, which is described in more detail

elsewhere [34]. The IRS-1 *Gly*972*Arg*, the TNF-α -308G/A, and the IL6 -174G/C polymorphisms were determined as described previously [32,35].

Statistical analysis

Variables with a skewed distribution (PAI-1 antigen, insulin, triglycerides, glucose, CRP), were logarithmically (ln) transformed prior to analysis, and they are presented as geometric means and SDs. Pearson correlation coefficients were calculated between PAI-1 concentrations and the metabolic variables and CRP levels. Differences between subgroups were analyzed with analysis of variance, after adjustments for only age and sex (simple model), and after adjustments for age, sex, the metabolic syndrome (WHR, insulin and triglycerides were taken as characteristics), and the other environmental factors (smoking, alcohol intake, physical activity, BMI) (extensive model). Differences between genotype groups were analyzed similarly (ANOVA with adjustments as in the simple model, or the extensive model). An interaction term in this model was used to evaluate possible interactions between environmental and genetic variables. A p-value smaller than 0.05 was considered statistically significant. All analyses were performed using SPSS 11.5 for Windows.

RESULTS

Table 1. Baseline characteristics of the study population

		Mean (±SD) n=561
Age (yr)		52.7 (±10.0)
Men (%)		44.90%
BMI (kg/m^2)		26.6 (±5.2)
WHR		0.87 (±0.11)
PAI-ag (ng/ml) ‡		78.7 (±31.6)
Insulin (pmol/l) ‡		42.8 (±7.3)
Triglycerides (mmol/l) ‡		1.1 (±0.06)
Glucose (mmol/l) ‡		5.2 (±0.05)
HDL-cholesterol (mmol/l)		1.3 (±0.36)
CRP (mg/l) ‡		2.12 (±0.74)
Smoking (% yes)		28.20%
Alcohol use (%)	0 glass/day	24.40%
	0-1 glass/day	41.00%
	>1 glass/day	34.60%
Physical activity (% > 2 days/week)		64.40%

Because of the non-normal distribution, the geometric means (±SD) of the ‡ levels are shown.

Baseline characteristics

The baseline characteristics of the study population are shown in Table 1. The population included 252 men and 309 women aged 26 to 71 years. The geometric mean PAI-1 antigen level was 78.7 ng/ml, and the PAI-1 antigen levels were higher in men than in women (105.3 ng/ml versus 62.1 ng/ml, p<0.001). PAI-1 antigen levels were strongly and positively associated with age, BMI, waist-hip ratio (WHR), and with glucose, insulin, triglyceride and CRP levels, and were inversely associated with HDL-cholesterol levels (p<0.001 in all cases) (Table 2).

Table 2: Spearman correlation coefficients of PAI-1 antigen levels with metabolic and inflammatory parameters

	R	p
Age	0.32	<0.001
BMI	0.65	<0.001
WHR	0.63	<0.001
Glucose ‡	0.5	<0.001
Insulin ‡	0.48	<0.001
Triglycerides ‡	0.51	<0.001
HDL-cholesterol	-0.48	<0.001
CRP ‡	0.32	<0.001

PAI-1 antigen levels and ‡ levels were logarithmically (ln) transformed prior to analysis

Environmental factors and PAI-1 antigen concentrations

The PAI-1 antigen concentration was related to several environmental factors in our population. PAI-1 antigen levels were significantly lower in subjects that had a BMI below the mean (26.6 kg/m^2) compared to individuals with a BMI above the mean after adjustment for age and sex (51.1 (±13.2) ng/ml versus 122.0 (±31.3) ng/ml p<0.001) (Table 3a), and when additional adjustment for the metabolic syndrome (WHR, insulin and triglycerides) and the other environmental factors (smoking, alcohol intake, physical activity) were applied (67.1 (±20.5) ng/ml versus 94.1 (±28.5) ng/ml, p<0.001). In addition, PAI-1 concentrations were significantly higher in physically inactive subjects after adjustment for age and sex (92.7 (±29.0) ng/ml versus 70.9 (±22.6) ng/ml, p<0.001). When the metabolic syndrome (WHR, insulin and triglycerides) and the other environmental factors (smoking, alcohol intake, BMI) were taken into account, this relationship was maintained (85.8 (±15.2) ng/ml versus 76.1 (±13.9) ng/ml, p=0.032). Smoking and alcohol intake were not significantly associated with PAI-1 antigen levels in our population.

Table 3a: PAI-1 antigen levels according to environmental factors

		PAI-1 ag (ng/ml) §	p-value	PAI-1 ag (ng/ml) ¶	p-value
BMI (kg/ m²)	<26.6 (n=282)	51.1 (±13.2)	<0.001	67.1 (±20.5)	<0.001
	>26.6 (n=279)	122.0 (±31.3)		94.1 (±28.5)	
Phys. Act. (days/ week)	0-2 (n=190)	92.7 (±29.0)	<0.001	85.8 (±15.2)	0.032
	3-7 (n=343)	70.9 (±22.6)		76.1 (±13.9)	
Smoking	No (n=395)	81.4 (±25.7)	0.075	78.81 (±14.2)	0.68
	Yes (n=155)	71.2 (±23.1)		80.88 (±15.2)	
Alcohol consumption (glass/ day)	0 (n=132)	75.1 (±24.1)	0.6	71.4 (±13.0)	0.082
	0-1 (n=222)	80.7 (±25.5)		81.0 (±14.6)	
	>1 (n=187)	82.0 (±27.2)		83.3 (±15.6)	

Values are presented as geometric means (±SD). PAI-1 levels adjusted for age and sex (§), and PAI-1 levels adjusted for age, sex, the metabolic syndrome (WHR, insulin, triglycerides) and the other environmental variables tested (physical activity, smoking, alcohol consumption and BMI) (¶) are shown.

Table 3b: PAI-1 antigen levels according to genetic variation

		PAI-1 ag (ng/ml) §	p	PAI-1 ag (ng/ml) ¶	p
PAI-1 -675(4G5G)	4G4G (n=144)	87.8 (±28.1)	0.14	89.3 (±9.8)	0.011
	4G5G (n=307)	75.3 (±24.6)		77.8 (±13.5)	
	5G5G (n=110)	77.5 (±25.2)		71.4 (±12.4)	
IRS-1 *Gly 972Arg*	GG (n=335)	84.6 (±27.6)	0.008	83.8 (±15.0)	0.014
	GA/AA (n=226)	70.8 (±22.8)		73.3 (±12.9)	
TNF-α -308G/A	GG (n=386)	76.9 (±25.0)	0.45	77.1 (±13.2)	0.22
	GA (n=156)	81.9 (±26.7)		84.4 (±15.1)	
	AA (n=19)	92.7 (±30.0)		88.7 (±15.9)	
IL6 -174G/C	GG (n=209)	82.8 (±26.5)	0.37	81.9 (±14.3)	0.7
	CG (n=278)	78.3 (±25.3)		78.3 (±13.9)	
	CC (n=70)	71.24 (±23.2)		77.8 (±13.9)	

Values are presented as geometric means (±SD). PAI-1 antigen levels adjusted for age and sex (§), and PAI-1 antigen levels adjusted for age, sex, the metabolic syndrome (insulin, triglycerides, WHR) and environmental variables (physical activity, smoking, alcohol consumption and BMI) (¶) are shown.

Genetic variation and PAI-1 antigen concentrations

The relationships between PAI-1 plasma concentrations and several genetic factors were also investigated. After adjustment for age, sex, the metabolic syndrome (WHR, insulin and triglycerides) and the environmental factors (smoking, alcohol intake, physical activity, BMI), there was a significant relationship between the -675(4G/5G) polymorphism in the PAI-1 promoter and PAI-1 antigen levels, and higher PAI-1 antigen levels were associated with the 4G allele (4G4G: 89.3 (±9.8) ng/ml, 4G5G: 77.8 (±13.5) ng/ml, 5G5G: 71.4 (±12.4) ng/ml, p=0.011) (Table 3b). Concerning the IRS-1 *Gly*972*Arg* polymorphism, the PAI-1 antigen levels of subjects homozygous for the *Gly* allele were significantly higher than the PAI-1 antigen levels of carriers of the *Arg* allele after adjustment for age and sex (84.6 (±27.6) ng/ml versus 70.8 (±22.8) ng/ml, p=0.008), and also after additional adjustments for the metabolic syndrome (WHR, insulin and triglycerides) and environmental factors (smoking, alcohol intake, physical activity, BMI) (83.8 (±15.0) ng/ml versus 73.3 (±12.9) ng/ml, p=0.014). No significant relationships between PAI-1 antigen levels and the TNF-α -308G/A and the IL6 -174G/C polymorphisms were observed.

Table 4: Significance levels of gene-environment interactions on PAI-1 antigen levels

	Smoking	**Alcohol**	**Phys. act**	**BMI**
	Yes /no	**0, 0-1, >1**	**High/ low**	**High/ low**
	Adjusted for age and sex (§)			
PAI-1 -675(4G/5G)	0.04*	0.86	0.97	0.72
IRS-1 Gly972Arg	0.32	0.41	0.19	0.63
TNF-α -308G/A	0.2	0.63	0.17	0.86
IL6 -174G/C	0.29	0.33	0.09	0.61
	Extensive adjustments (¶)			
PAI-1 -675(4G/5G)	0.23	0.6	0.45	0.91
IRS-1 Gly972Arg	0.3	0.71	0.95	0.25
TNF-α -308G/A	0.4	0.71	0.05*	0.44
IL6 -174G/C	0.26	0.74	0.07	0.41

Models adjusted for age and sex (§), and adjusted for age, sex, metabolic syndrome and environmental factors (¶) are shown. * $p<0.05$

Gene-environment interactions

As it is biologically plausible that the effects of genetic variation may be particularly relevant under specific conditions, interactions between the environmental factors (smoking, alcohol intake, physical activity and BMI) and the genetic factors were investigated. A significant interaction between the PAI-

1 -675(4G/5G) polymorphism and smoking was observed (p=0.04), after adjustment only for age and sex (Table 4). However, this interaction disappeared when additional adjustments were made for the metabolic syndrome (WHR, insulin and triglycerides) and the other environmental factors (alcohol intake, physical activity, BMI) (p=0.23). In addition, a significant interaction was observed between the TNF-α -308G/A polymorphism and physical activity after adjustments for age, sex, the metabolic syndrome (WHR, insulin and triglycerides) and the other environmental factors (smoking, alcohol intake, BMI) , (p=0.05), and the PAI-1 antigen levels were increased in physically inactive subjects homozygous for the TNF-α-308A allele (Figure 1). This interaction was not present when only age and sex were taken into account (p=0.17).

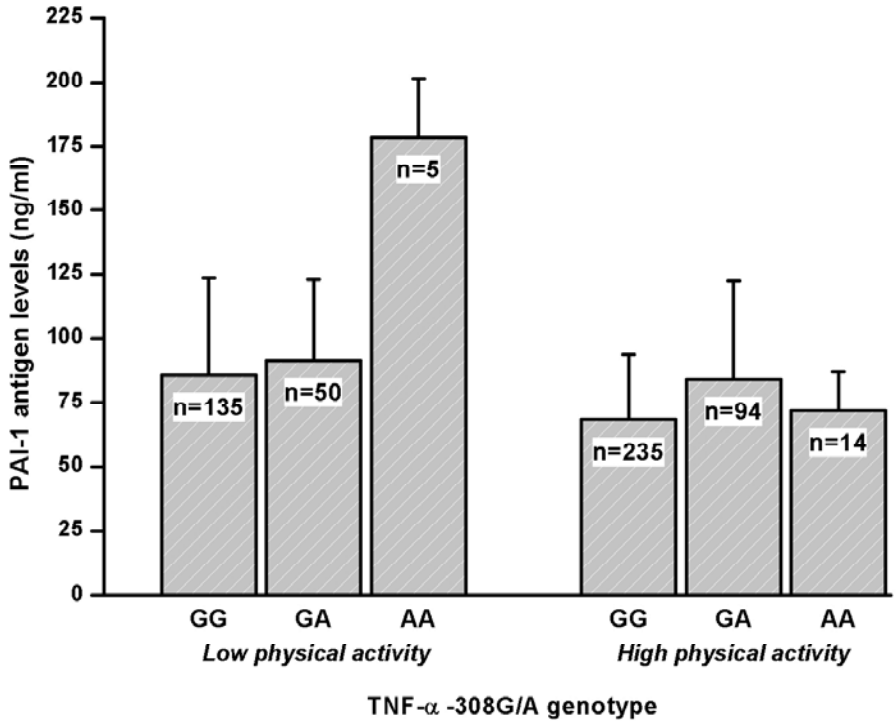

Figure 1: PAI-1 antigen levels of the different TNF-α-308G/A genotypes, in physically inactive and physically active subjects. Bars represent the geometric means (±SD) of the PAI-1 antigen levels. The number of subjects in the subgroups is indicated on the bars.

DISCUSSION

Elevated PAI-1 plasma levels are associated with an increased risk of cardiovascular disease [7,8]. In this study we describe the relationship between several environmental and genetic factors, and PAI-1 antigen levels. We focused

on the metabolic syndrome and inflammation, as both these biological processes are important for PAI-1 concentrations and also for cardiovascular disease. We were also interested in identifying factors that influenced PAI-1 concentrations independently of the metabolic syndrome.

We have observed tight correlations between PAI-1 antigen levels and several features of the metabolic syndrome, e.g. BMI, WHR, glucose levels, insulin levels, triglyceride levels, and HDL cholesterol levels. This confirms that PAI-1 antigen levels are related to the metabolic syndrome, and this conclusion is in accordance with the literature [18,36]. In addition we observed a tight correlation between PAI-1 antigen levels and the sensitive acute-phase marker CRP, and with this finding we confirm the relationship between PAI-1 and inflammation that had been suggested before [22,37]. PAI-1 transcription is under direct control of several cytokines including TNF-α and transforming growth factor-β (TGF-β). Elevated plasma levels of these cytokines are also a feature of the metabolic syndrome [23,38], which might explain the interrelationships between the metabolic syndrome, inflammation and PAI-1 plasma concentrations [22,39]

In this study, we evaluated the relationships between PAI-1 concentrations and the environmental factors BMI, physical activity, smoking and alcohol intake. First of all, we observed significantly higher PAI-1 concentrations in subjects with a BMI above the mean, which was expected based on the results of others [18,21]. We observed that subjects that were physically active had lower PAI-1 concentrations than subjects that were inactive ($p<0.001$ adjusted for age and sex, and $p=0.032$ after adjustment also for the metabolic syndrome and the other environmental factors). In a previous study, it was shown that after a six-month intervention in which the participants underwent a controlled exercise program, PAI-1 levels were moderately decreased. Our results support this conclusion that regular physical exercise has a beneficial, reducing effect on PAI-1 plasma concentrations. [40]. We did observe slightly higher PAI-1 antigen levels in non-smokers compared to smokers, and this might be explained by the fact that in general non-smokers have a higher BMI than smokers, which also was the case in our population (the average BMI was 27.3 in non-smokers and 24.5 in smokers, $p<0.001$). This is underlined by the fact that after additional adjustment for the metabolic syndrome and the other environmental factors (including BMI), the PAI-1 antigen levels in non-smokers and smokers were similar (Table 3a). Finally, we did not observe a strong relationship between PAI-1 concentrations and alcohol intake, although PAI-1 antigen levels seemed to increase with increasing alcohol consumption, which is in line with the results of others [41], and this elevated PAI-1 production might be related to increased cytokine production in the early stage of many liver diseases, including alcoholic steatohepatitis [42]. However, our results may also rely on chance as they were not significant and in some other studies no clear relationship between PAI-1 concentrations and alcohol intake could be detected [43]. Among the environmental factors that we investigated, BMI was the

strongest effector of PAI-1 antigen levels, also independent of the other environmental factors (smoking, alcohol intake, physical activity), and the metabolic syndrome (WHR, insulin and triglycerides) (67.1 (±20.5) ng/ml versus 94.1 (±28.5) (ng/ml, $p<0.001$). The PAI-1 antigen levels of subjects with a BMI above the mean was 1.4 fold increased compared to the PAI-1 antigen levels of subjects with a BMI below the mean. This indicates that the relationship between PAI-1 plasma concentrations and BMI might be independent of the other characteristics of the metabolic syndrome, and this is an interesting observation that should be confirmed in other studies.

We also investigated the relationship between several genetic factors and PAI-1 antigen levels, and also in this analysis we were interested in the effect of the metabolic syndrome on these relationships. Generally, the 4G allele of the -675(4G/5G) polymorphism in the PAI-1 gene is associated with elevated PAI-1 levels [27-29]. Also in our population, higher PAI-1 antigen levels were observed associated with the -675(4G) allele, and this relationship was significant when adjustments were made for age, sex, the metabolic syndrome and environmental factors ($p=0.011$). This indicates that in our population the -675(4G/5G) polymorphism influences PAI-1 antigen levels, but the metabolic syndrome and environmental factors need to be taken into account in order to detect this relationship, which is in line with other studies [29,36]. Our results could indicate that BMI and the -675(4G/5G) variation influence PAI-1 antigen levels independently, and that in order to detect the relatively small effect of the -675(4G/5G) polymorphism, adjustment for BMI needs to be made. We have obtained similar results in another population of healthy Caucasians as well, and this has been described elsewhere (**chapter 5**). Secondly, we observed lower PAI-1 plasma levels in carriers of the rare *Arg* allele of the *Gly*972*Arg* polymorphism in the insulin-receptor substrate-(IRS)-1 gene, which is in line with findings of Clausen *et al* [19]. However, this relationship disappeared when the metabolic syndrome (WHR, insulin and triglycerides) and environmental factors (smoking, alcohol intake, physical activity) were also considered, indicating that the effect of the IRS-1 polymorphism on PAI-1 levels might work through the metabolic syndrome. This is biologically plausible, as IRS-1 is important in the cellular response to insulin, and insulin is a key player in the metabolic syndrome. In this population, carriers of the rare IRS-1 972*Arg* allele indeed have higher insulin levels, particularly when they are also overweight [32]. Therefore, higher insulin levels in carriers of the IRS-1 972*Arg* allele are a plausible explanation for the relationship between the IRS-1 972*Arg* allele and elevated PAI-1 antigen levels that we describe in the present work. We did not observe significant relationships between the functional TNF-α -308G/A and IL6 -174G/C polymorphisms and PAI-1 antigen levels, although in both cases there was a trend towards higher PAI-1 antigen levels in the carriers of the alleles associated with high cytokine production (the -308A allele of the TNF-α polymorphism, and the -174G allele of the IL6 polymorphism [44,45]). However,

the relevance of these two polymorphisms for PAI-1 plasma levels in the general healthy population is probably limited.

It is biologically plausible that effects of genetic variation are particularly relevant under specific (environmental) conditions. Therefore we have investigated the interactions between the environmental factors and genetic factors, on PAI-1 concentrations. We observed two possible interactions that deserve further discussion. Firstly, we observed an interaction between the PAI-1 -675(4G/5G) polymorphism and smoking status after adjustment for age and sex only, but this interaction disappeared when also the environmental and metabolic factors were taken into account. This can be explained by the fact that smoking often coincides with low BMI, suggesting that this interaction does not influence PAI-1 concentrations independently of other metabolic and environmental factors. Therefore, this interaction may be less relevant for PAI-1 plasma concentrations from a biochemical point of view. Secondly, we observed an interaction between the TNF-α -308G/A polymorphism and physical activity on PAI-1 antigen levels after adjustment for age, sex, the metabolic syndrome and the other environmental factors. This interaction is biologically possible, as it has been shown that physical activity can reduce inflammation [46]. We observed that the PAI-1 antigen levels in physically inactive subjects homozygous for the TNF-α-308A allele were approximately 2.2 fold higher than the PAI-1 antigen levels in others. The TNF-α-308A allele is associated with increased TNF-α promoter activity *in vitro* [44]. Our results suggest that the effect of this polymorphism on TNF-α expression might be strong enough to influence PAI-1 expression under the relatively pro-inflammatory conditions present in physically inactive subjects. However, this hypothesis is based on a small number of subjects in this subgroup, and this association should therefore be verified in other studies.

In summary, this study describes effects of several environmental and genetic factors on PAI-1 antigen levels. In this population, BMI, physical activity and the PAI-1 -675(4G/5G) and the IRS-1 Gly972Arg polymorphisms affect PAI-1 levels independent of the metabolic syndrome. In addition, we observed indications for an interactive effect between the TNF-α -308G/A polymorphism and physical activity on PAI-1 plasma concentrations.

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Chapter 7

General Discussion and Conclusions

THIS CHAPTER

The overall aim of the studies described in this thesis was to investigate the effects of common genetic variations in the promoter regions of the fibrinogen β and plasminogen activator inhibitor-1 (PAI-1) genes on promoter activity and plasma protein levels. This chapter contains the general discussion of issues concerning the functional studies of the fibrinogen β promoter (**chapter 2 and 3**), and a discussion of the studies on the relationship between PAI-1 promoter variation and PAI-1 antigen levels (**chapter 5 and 6**). Additionally, some general issues concerning the role of genetic variation in the promoter regions of genes (**chapter 4**) and its relationship with cardiovascular disease are discussed.

REGULATION OF FIBRINOGEN β CHAIN GENE EXPRESSION

In **chapter 2** we describe the identification of a functional hepatocyte nuclear factor-3 (HNF-3) element located at position -159/-151 in the fibrinogen β promoter. Promoter-reporter gene assays showed that this site plays an important role in the interleukin-6 (IL6) response of the fibrinogen β gene, and supershift assays demonstrated that in HepG2 nuclear extracts, HNF-3β did bind to this sequence under basal and IL6-induced conditions. In addition, we observed that the IL6-responsive CCAAT box/ enhancer-binding protein (C/EBP) site at -133/-125 in the fibrinogen β promoter interacts with the novel HNF-3 element, which is a plausible explanation for the importance of this HNF-3 element in the IL6 response of the fibrinogen β promoter.

Interestingly, inspection of the promoter regions of genes encoding other acute-phase proteins, revealed that the promoters of the fibrinogen α and C-reactive protein (CRP) genes also contain a putative HNF-3 site located adjacent to IL6-responsive C/EBP elements (MatInspector Professional) [1,2]. This suggests that a regulatory pathway involving HNF-3 and C/EBP might be important in the IL6 response of a number of acute phase genes, and this increases the relevance of a detailed understanding of the role of HNF-3 in the acute phase response of the fibrinogen β gene.

With our identification of the HNF-3 element at -159/-151 in the fibrinogen β promoter, three major elements important in the IL6 response of this gene have now been described: the HNF-3 site, the C/EBP site and the IL6 responsive element (IL6 RE). The IL6 RE is located at position -143/-137, between the HNF-3 and the C/EBP sites, and its role is not yet understood [3,4]. For a good understanding of the mechanism of the IL6 response of the fibrinogen β gene and the role of the HNF-3 site, the functioning of the IL6 RE needs to be clarified. Several hypotheses concerning the function of the IL6 RE will be

discussed in the following paragraphs, including their implications for the role of HNF-3.

First of all, the sequence of the IL6 RE represents a perfect half-site for the IL6-activatable transcription factor signal transducer and activator of transcription-3 (STAT3) [5], and therefore the most straightforward hypothesis is that STAT3 binds this IL6 RE in the fibrinogen β promoter. Surprisingly, binding of STAT3 to the IL6 RE or transactivation of the fibrinogen β promoter by overexpression of STAT3 could not be detected, neither in the presence nor in the absence of IL6 (Gervois, personal communication and our unpublished data). However, it is possible that STAT3 requires the presence of other transcription factors, and therefore no effect of STAT3 may be detectable in experiments that focus on the isolated effect of STAT3. This theory is supported by the results of Duan *et al*, which show that STAT3 does bind weakly to the identical IL6 REs in the fibrinogen γ promoter, and that other nuclear factors are also present in the transcriptional complex [6]. The presence of both STAT3 and HNF-3 in the same transcriptional complex is plausible, as direct interaction of these two transcription factors has been reported [7]. However, an argument against the involvement of STAT transcription factors in the regulation of the fibrinogen β promoter may be that the IL6 RE in the fibrinogen β promoter does not represent a complete STAT site but only a half-site. Until now, it had been thought that STAT transcription factors bind as dimers, and consequently should require a complete STAT site instead of a half-site.

Therefore, a second hypothesis is that the IL6 RE does not bind a transcription factor of its own and that its similarity to a STAT half-site is a coincidence. Then the IL6 RE, which is located between the HNF-3 and C/EBP binding sites, may only be required to correctly position HNF-3 and C/EBPβ in relation to each other. Mutations in the IL6 RE sequence might slightly change the position of the HNF-3 and C/EBPβ sites in relation to each other, resulting in a decreased interaction between these two transcription factors, explaining the loss of IL6 response observed after the mutation of the IL6 RE [3,4]. If indeed the IL6 RE does not bind a transcription factor of its own, HNF-3 and C/EBP may interact either directly or indirectly through an intermediating protein, which does not bind to the DNA itself, but does physically interact with HNF-3 and C/EBP. Then again, there are also arguments that oppose the above-mentioned hypothesis, and that do suggest binding of a specific transcription factor to the IL6 RE. For instance, the three fibrinogen chains are coordinately expressed during the acute-phase response, and functional IL6 REs with identical sequences have been identified in the promoter regions of all three fibrinogen genes. However, the organization of the regulatory elements in the vicinity of the IL6 REs is different. A functional C/EBP site and a putative HNF-3 site are also present in the fibrinogen α promoter, but not in the fibrinogen γ promoter. In addition, in the fibrinogen β promoter the IL6 RE is located between the C/EBP and HNF-3 sites, whereas in the fibrinogen α promoter the IL6 RE is

located just downstream of the C/EBP and HNF-3 sites [1,8]. Identical sequences but different locations relative to other regulatory elements in the three fibrinogen promoters, argue against the hypothesis that the IL6 RE is only required for the positioning of other regulatory elements, but suggest that there is a specific factor binding the IL6 REs.

Therefore, a third hypothesis is that the IL6 RE in the fibrinogen β promoter does bind a specific transcription factor other than STAT3. As HNF-3 has been shown to interact with a variety of different transcription factors including C/EBPβ, STAT3, HNF-4, and p53 [7,9,10], HNF-3 may also interact with this putative transcription factor binding to the IL6 RE.

POLYMORPHISMS IN THE FIBRINOGEN β PROMOTER

Six polymorphisms have been described in the proximal 2000 bp of the fibrinogen β promoter: the -1420G/A, -993C/T, -854G/A, -455G/A, the -249C/T and the -148C/T variations. The -1420G/A variation is in (nearly) complete linkage disequilibrium with the -993C/T, -455G/A and -148C/T variations in Caucasians [11]. Our study shows that the change from C to T at position -148 decreases basal and IL6-induced fibrinogen β promoter activity by influencing the adjacent HNF-3 site and its interaction with the C/EBP element. This explains the lowering effect on promoter activity of this nucleotide change, at the molecular level (**chapter 3**).

Identification of the functional fibrinogen β promoter variation has also been the aim of functional promoter-reporter gene studies from several other groups, and the results of these studies are not always in accord with ours. Using promoter fragments of 640 bp of the proximal fibrinogen β promoter cloned in a reporter gene vector, Brown *et al* showed that a change from G to A at position -455 resulted in increased basal and IL6-induced expression [12]. A year later, van 't Hooft *et al* demonstrated with fragments of 1100 bp of the proximal fibrinogen β promoter cloned in a reporter gene vector, that the -854A (unique for haplotype b) and -455A (unique for haplotype d) alleles were associated with increased basal fibrinogen β promoter activity [13], whereas in our experiments with 1800 bp fibrinogen β promoter fragments, only the -148C/T variation affected basal and IL6-induced fibrinogen β promoter activity (**chapter 3**). In another study, functional effects were indicated for the -1420G/A polymorphism, and the involvement of a putative HNF-3 element located just downstream of the -1420G/A polymorphism was suggested. In this study, Wragg *et al* showed with promoter-reporter gene assays including 1480 bp fibrinogen β promoter fragments that the -1420G/A polymorphism influenced the response of the promoter to HNF-3 [14]. Also in our experiments HNF-3 turned out to be a key player, and therefore we extended our experiments and also investigated the isolated effects of the -1420G/A

polymorphism. In addition to the fibrinogen β promoter haplotype constructs described in **chapter 3**, we created haplotype constructs that differed only at position -1420G/A from their natural counterparts. Using these constructs, we could not detect any effect of the G to A change at position -1420 on basal, IL6-induced, or HNF-3-induced fibrinogen β promoter activity in our assay system (data not shown). We also mutated the putative HNF-3 consensus sequence located at -1418/-1410, just downstream of the -1420G/A variation by disrupting the core sequence, analogous to the mutant promoter-reporter gene constructs that we created to investigate the HNF-3 site adjacent to the -148C/T polymorphism (**chapter 2**). Mutation of the putative HNF-3 site at -1418/-1410 did not affect basal, IL6-induced, or HNF-3-induced fibrinogen β promoter activity, indicating that in our assay system the putative HNF-3 site at -1418/-1410 is not an important regulatory element (data not shown).

In conclusion, some controversy still exists about the functional effects of the different variations in the fibrinogen β promoter. This may partly be explained by differences in experimental design for instance with respect to the length of the fibrinogen β promoter fragments used. However there are three reasons why we believe that we have strong evidence for a functional role of the -148C/T variation. Firstly, decreased IL6-induced and basal fibrinogen β promoter activity by a change from C to T at position -148, has been confirmed by others [11,15]. Secondly, in ethnic groups with incomplete linkage disequilibrium between the -455G/A and -148C/T variations (both located on haplotype d), a stronger association was seen between -148C/T and plasma fibrinogen levels than between -455G/A and plasma fibrinogen levels [16]. Thirdly, following the identification of the -148C/T variation as the functional variation, we have been able to explain the effects of this variation on fibrinogen β promoter activity, at the molecular level. Therefore, we suggest that the -148C/T variation is selected to investigate genetic variation of the fibrinogen β gene in epidemiological studies.

FIBRINOGEN β PROMOTER ACTIVITY IN RELATION TO PLASMA FIBRINOGEN LEVELS

With our *in vitro* luciferase assays we identified the -148C/T polymorphism as the functional variation in the fibrinogen β promoter, and we observed that the T allele of the -148C/T polymorphism was associated with decreased IL6-induced and basal promoter activity, which has also been observed *in vitro* by others [11,14]. However, in population-based association studies, the -148T allele generally is related to elevated basal and acute phase-induced plasma fibrinogen levels (**chapter 3**) [17-20]. This apparent discrepancy between *in vitro* and *in vivo* findings is puzzling, and will be further discussed in the paragraphs below.

There are several technical issues, peculiar to the *in vitro* experiments as we performed them, which may have contributed to this *in vivo - in vitro* discrepancy. Firstly, we chose a hepatoma cell line (HepG2) as model system, since no relevant sources of fibrinogen other than the liver had been described. A reduced basal and IL6-induced promoter activity associated with the -148T allele has been observed in two additional hepatoma cell lines (HuH7 and Hep3B) by others and by us, and therefore it is unlikely that the cell types used were responsible for the inconsistency with the results from *in vivo* association studies [14]. Secondly, the situation in the human body is different from the conditions in our cell cultures. For instance, we used only IL6 to mimic the acute phase response, but in the case of an acute-phase event *in vivo* several inflammatory mediators besides IL6 are expressed. Different inflammatory mediators will activate different transcription factors that can have opposite effects, especially when these factors bind to adjacent or overlapping DNA elements. In this respect, our inadequate knowledge of the IL6 RE in the fibrinogen β promoter may be a complicating factor. The IL6 RE is located between the HNF-3 and the C/EBP sites; a putative third transcription factor binding the IL6 RE might compete for binding with HNF-3 or C/EBPβ. Such competition has been described on several occasions, and it often represents a mechanism of inhibition of gene induction [9,21]. As the mechanism of the IL6 RE is still unclear, and the -148C/T polymorphism is located right between the IL6 RE and the HNF-3 site, the net effect of the polymorphism on fibrinogen β promoter activity can probably only be fully explained if the IL6 RE is completely understood. A third issue concerning the experimental conditions is that *in vivo* interaction between hepatocytes and other cell types could be important, whereas these interactions were absent in our HepG2 tissue cultures. A fourth technical point to consider is the DNA fragment selected. We used a 1800 bp fragment of the fibrinogen β promoter because a fragment of this size is expected to include all major regulatory sequences, and because larger DNA fragments become increasingly unpractical. This means that there is always a risk of missing important regulatory elements. For instance, the sequences responsive to glucocorticoids have been mapped between -2900 and -1500 relative to the transcription start site in the fibrinogen β promoter, but their exact location is not known [22]. Therefore, these responsive elements may not have been present in our 1800 bp fragments. This could be relevant because of the synergistic effect that glucocorticoids and IL6 have on fibrinogen expression [23]. To summarize, the *in vitro* model system is simpler in design than the naturally occurring systems in the human body, and this may have contributed to the discrepancy between our *in vitro* results and the *in vivo* results published by others.

An alternative explanation is that another functional polymorphism located outside the promoter region, but in linkage disequilibrium with the -148C/T polymorphism, is the actual cause of the increased fibrinogen levels associated with the fibrinogen β promoter haplotype d (characterized by -455A and -148T)

in association studies. The relevance of linkage disequilibrium is illustrated by the following example. On some occasions, a relationship between the promoter haplotype d (including the -455A and -148T alleles) and an increased risk of MI has been reported, which has been attributed to the higher plasma fibrinogen levels associated with the haplotype d [24,25]. However, the promoter haplotype d extends into the coding region of the gene and is also characterized by the rare allele of the 448Arg/Lys polymorphism [26]. Substitution of the arginine for a lysine at position 448 in the fibrinogen β molecule results in thinner fibrin fibres and a more tightly packed clot, and it has been proposed that this change in clot structure might influence the clinical outcome [27]. Although the relevance of the 448Arg/Lys polymorphism is not yet clear, and the relationship between the haplotype d and the risk of MI is far from certain, this example shows that different polymorphisms unique to the same functional haplotype (-455G/A and -148C/T versus 448Arg/Lys) can potentially influence the same events (e.g. MI) through different mechanisms (increased plasma levels versus clot structure).

The issues discussed in the preceding paragraphs underline the difficulty of extrapolating results from functional *in vitro* assays on the effect of a single polymorphism, and applying them to the results of population-based association studies that represent the net result of numerous genetic and environmental factors. Epidemiological association studies are powerful in detecting associations and therefore they can offer a framework for the interpretation of results obtained in *in vitro* experiments and in animal studies, but they cannot provide evidence of the underlying molecular mechanisms. For this, *in vitro* experiments and animal experiments are necessary, even though they also have their limitations. Multiple model systems should then be used to tackle research questions from different angles, and together they should help to elucidate the biological mechanisms.

FUTURE RESEARCH ON THE FIBRINOGEN β CHAIN GENE PROMOTER

On the basis of the results described in **chapters 2 and 3** of this thesis, there are several research lines that can be investigated further.

Firstly, it would be interesting to investigate whether HNF-3 is a general regulator of the acute phase response. The presence of putative HNF-3 sites in the vicinity of functional C/EBP elements also in the promoter regions of other acute-phase genes, could indicate that the cooperation between HNF-3 and C/EBP in the IL6 response is a more general mechanism regulating the (hepatic) acute phase response (**chapter 2**). In addition, further understanding of the cooperation between C/EBP and HNF-3 in the IL6-mediated regulation

of the fibrinogen β gene will be increasingly relevant if indeed this cooperation turns out to be a general mechanism regulating the acute phase response.

Furthermore, for a complete picture of the regulation of IL6-induced fibrinogen β expression by HNF-3 and C/EBP, and the effect of the -148C/T variation, the role of IL6 RE in the fibrinogen β promoter needs to be clarified.

As a secondary aim, the effects of fibrates can also be investigated in the experiments suggested in this section. These hypolipidaemic and anti-inflammatory drugs specifically reduce plasma levels of fibrinogen by decreasing the activity of the C/EBP element, and the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) plays a crucial role in this mechanism [28,29]. Research into the role of the IL6 RE and the HNF-3 site in the interaction of PPARα with the C/EBP element, could provide further understanding of the mechanism by which fibrates influence plasma protein levels. Finally, also the effect of the -148C/T polymorphism should be addressed in these experiments as it is likely to modulate these events.

GENETIC VARIATION OF THE PAI-1 GENE AND PLASMA PAI-1 LEVELS

We have observed that PAI-1 promoter haplotypes, determined by the -844A/G and the -675(4G/5G) polymorphisms, are associated with PAI-1 antigen levels, most prominently in lean individuals. In these lean subjects, the PAI-1 antigen levels of A-4G/G-5G heterozygotes were reduced to approximately 60-75% and the G-5G homozygotes to approximately 45-55%, compared to the PAI-1 antigen levels of A-4G homozygotes. However, we also observed that the effect of BMI on PAI-1 concentrations was much stronger than the effect of PAI-1 promoter variation, as PAI-1 plasma levels increased approximately fourfold from the lowest to the highest BMI quartile (**chapter 5**). To our knowledge this is the first study that has investigated the relationship between PAI-1 promoter variation and PAI-1 antigen levels in quartiles according to BMI. However, our conclusion that metabolic factors (e.g. BMI) are more important determinants of PAI-1 levels than genetic variation, is supported by others [30].

It is interesting to hypothesize how our observation that PAI-1 promoter variation is relevant for PAI-1 antigen levels in lean individuals, connects to the reported relationships between PAI-1 promoter variation, PAI-1 antigen levels and cardiovascular risk. In general, the reported relationships between PAI-1 promoter variation and PAI-1 antigen levels, and between PAI-1 antigen levels and cardiovascular risk are consistent, but the relationship between PAI-1 promoter variation and cardiovascular risk is much weaker [31,32]. Our study demonstrates that the influence of genetic variation on PAI-1 antigen levels is primarily relevant in individuals with low BMI, which means that these individuals have a favourable status with respect to this cardiovascular risk

factor. The relevance of genetic variation to PAI-1 levels primarily in a subgroup in an advantageous position to other (risk) factors, might explain the weak relationship between the genetic variation of the PAI-1 promoter and the risk of cardiovascular disease.

GENETIC VARIATION OF THE PAI-1 PROMOTER UNDER BASAL CONDITIONS *IN VITRO*

One aim of this thesis was to identify the functional polymorphism(s) in the promoter region of the PAI-1 gene. In the MORGEN and Glostrup populations we observed higher PAI-1 levels associated with the -844A/-675(4G) haplotype, and lower PAI-1 antigen levels associated with the -844G/-675(5G) haplotype (**chapter 5**). However, due to the low number of the other haplotypes in our populations, we could not discriminate between the effects of the -844A/G and the -675(4G/5G) polymorphisms. To identify the functional polymorphism in the PAI-1 promoter and to elucidate the underlying molecular mechanisms, we performed *in vitro* promoter-reporter gene assays with the four different PAI-1 promoter haplotypes. In these assays we could not detect a direct effect of PAI-1 promoter variation on basal PAI-1 promoter activity in hepatoma or endothelial cells, and these *in vitro* observations are consistent with the *in vitro* findings under basal conditions of others (Schoenhard *et al* personal communication) [33,34]. However, we did observe a relationship between PAI-1 promoter variation and PAI-1 antigen levels in our *in vivo* association studies (**chapters 5 and 6**), and the apparent discrepancy with the *in vitro* findings could be caused by technical limitations in our experimental model, similar to the ones listed in the previous section on the fibrinogen β promoter. Another possible explanation is that the actual functional PAI-1 polymorphism is located outside the promoter region that we included in the functional assays. In that case, the PAI-1 promoter haplotype would only be a marker of this functional polymorphism. PAI-1 expression is highly regulated at the post-transcriptional level by sequences located at the 3' end of the gene, and it has been shown previously that changes in the 3' sequence of the gene can affect mRNA stability [35-38]. In the 3' untranslated region (UTR) of the PAI-1 gene several variations are present, including the +10707C/T and the 11053G/T polymorphisms. These two variations are both in nearly complete linkage disequilibrium with the promoter polymorphisms, and could be very interesting candidates for further investigation (http://pga.gs.washington.edu/data/serpine1/) [39]

GENETIC VARIATION OF THE PAI-1 PROMOTER UNDER INDUCED CONDITIONS *IN VITRO*

It has been reported that the relationship between PAI-1 concentrations and plasma triglyceride levels is modified by the genetic variation of the PAI-1 gene, indicating that the regulation of PAI-1 expression might be influenced by genotype [40,41]. Since then, many *in vitro* studies have been performed, investigating the influence of the -675(4G/5G) polymorphism on the response of the PAI-1 promoter to a variety of inducers. In these investigations, either the endogenous PAI-1 promoter or transfected PAI-1 promoter fragments were studied in several different cell types (endothelial cells, adipocytes, hepatocytes, smooth muscle cells). In some of these studies, the response of the PAI-1 promoter to interleukin-1 (IL1), transforming growth factor-β (TGF-β), very-low-density lipoprotein (VLDL) and insulin, was dependent on the -675(4G/5G) genotype [34,42,43]. However, additional studies have not been able to confirm these effects of the -675(4G/5G) polymorphism on the response of the PAI-1 promoter (Schoenhard *et al* personal communication and our unpublished data) [33,34,44-46]. Because of the conflicting results it is unclear which inducer stimulates PAI-1 expression genotype-dependently, if any.

A relationship between the -675(4G/5G) polymorphisms and the circadian variation of PAI-1 plasma concentrations levels has also been suggested. Two epidemiological studies have reported that the circadian oscillation of PAI-1 antigen is more prominent in carriers of the -675(4G) allele than in -675(5G) homozygotes [47,48]. In addition, it has been shown *in vitro* that the circadian oscillation of PAI-1 expression is mediated by an E-box element located just upstream of the -675(4G/5G) polymorphism [49]. However, when these *in vitro* experiments were repeated with the -675(4G/5G) polymorphism taken into account, no effect of the polymorphism on the transcriptional regulation of the circadian expression could be detected (Schoenhard *et al* personal communication). Therefore, it is not certain whether the more pronounced circadian oscillation observed in -675(4G) carriers is caused by the -675(4G/5G) polymorphism itself, or that the oscillation is merely more evident in -675(4G) carriers as they generally have higher PAI-1 plasma levels than -675(5G) homozygotes.

FUTURE RESEARCH ON GENETIC VARIATION OF THE PAI-1 PROMOTER

First of all, our conclusion that the relationship between PAI-1 promoter haplotype and PAI-1 plasma levels is most prominent in lean individuals deserves to be re-established in other populations, although the relevance of the genetic variation of the PAI-1 gene for cardiovascular disease is probably limited. Concerning functional *in vitro* studies, more research into the

functional effects of the PAI-1 promoter polymorphisms does not seem relevant at this moment, as we did not find clear indications of a functional effect of the -844A/G and -675(4G/5G) polymorphisms on PAI-1 promoter activity. On the other hand, the regulation of PAI-1 mRNA levels by sequences located at the 3' end of the PAI-1 gene is a relatively unexplored research area that is worth further investigation. In such studies, the polymorphisms located in the 3' region of the PAI-1 gene should be taken into account. If indeed these polymorphisms do affect PAI-1 expression levels, they might explain the association between PAI-1 promoter variation and PAI-1 antigen levels observed *in vivo*.

DIFFERENTIAL EFFECTS OF GENETIC VARIATION ON GENE EXPRESSION

The effects of genetic variation on gene expression can vary greatly among the different genes. The fibrinogen β and PAI-1 genes, which are described in this thesis, vary in this respect.

For fibrinogen, the most important inducer is IL6. We show with functional assays that genetic variation of the fibrinogen β promoter interacts with the IL6 response of the gene (**chapter 3**). In addition, it has been reported that in the presence of a strong acute-phase trigger, the average plasma fibrinogen level was ±12%, ±15% and ±30% higher in carriers of the -455A (and -148T) allele compared to -455GG (and -148CC) homozygotes, in groups of 250, 45 and 60 individuals respectively. This indicates that fibrinogen β promoter variation can be relevant for plasma fibrinogen levels, particularly in the presence of a strong acute-phase stimulus [19,20,50].

In the case of PAI-1, the situation is different. Our study shows that genetic variation is relevant for PAI-1 concentrations, as we observed that PAI-1 antigen levels are approximately 50% lower in G-5G homozygotes than in A-4G homozygotes. However, this was confined to lean individuals, and in more obese subjects the effect of genetic variation on PAI-1 concentrations is easily overruled by the effect of BMI (**chapter 5**). This can be explained by the way the PAI-1 gene is regulated. PAI-1 expression is under the influence of many (metabolic syndrome-related) substances, including insulin, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), VLDL, fatty acids, glucose, angiotensin II, and hypoxia. These inducers regulate PAI-1 expression by distinct mechanisms through response elements mostly located at different sites in the PAI-1 promoter [44,51-57]. Therefore, the effect of genetic variation of the PAI-1 promoter on PAI-1 expression is probably limited, because of the overriding signals of several inducers. This is supported by our results described in **chapter 5**, showing that the effect of genetic variation on PAI-1 levels is less prominent in subjects with increased BMI, which are the

individuals who generally have increased levels of the many factors associated with the metabolic syndrome.

These examples of fibrinogen and PAI-1 show that the relevance of genetic variation to gene expression and plasma protein levels is dependent on the importance of the genotype-dependent trigger relative to other triggers, and on the mechanism by which genetic variation influences the plasma protein levels.

Another possibility is that the genetic variation of regulating factors influences the expression of the gene of interest. In this thesis, two examples of this principle are indicated. Firstly, in **chapter 4** we show that the response to vaccination by CRP plasma levels *in vivo*, is influenced by the -174G/C polymorphism in the promoter region of its inducer; IL6. Secondly, in **chapter 6** we show that the *Gly*972*Arg* polymorphism in the insulin-receptor substrate-1 (IRS-1) gene, which is important in the cellular response to insulin, is associated with PAI-1 antigen levels. However, we need to make provisos in both these cases. Firstly, the effect of the IL6 -174G/C polymorphism on the response of CRP levels was statistically significant and therefore detectable, but fairly small with respect to the absolute effect on plasma levels. In the case of the IRS-1 *Gly*972*Arg* polymorphism, only a few subjects (1.8% of the population) were homozygous for the rare *Arg* allele, therefore they were pooled and analysed together with the *Gly/Arg* heterozygotes. However, the PAI-1 antigen levels of the *Arg/Arg* homozygotes were in fact lower than those of *Gly/Arg* heterozygotes, whereas assuming an allele-dosage effect, their levels were expected to be higher than those of *Gly/Arg* heterozygotes. Therefore, although the biological mechanisms in both these examples are plausible, their effects may be small (in the case of the IL6 -174G/C polymorphism and CRP levels), and we might have to be careful with the interpretation of the results (in the case of the IRS-1 *Gly*972*Arg* polymorphism and PAI-1 levels).

RELEVANCE OF GENETIC VARIATION TO THE RISK OF CARDIOVASCULAR DISEASE

There is evidence that both PAI-1 and fibrinogen can directly contribute to cardiovascular disease, but increases in their plasma levels also coincide with incidence of the disease. Therefore, their association with cardiovascular risk could largely rely on the latter. Many biological processes are involved in the development of cardiovascular disease, including blood coagulation, fibrinolysis, inflammation, lipid metabolism, glucose metabolism, and blood pressure. Therefore, the importance for disease risk of a polymorphism with a mild or moderate effect on the plasma levels of a single factor in one of these several biological events, is expected to be small. In fact, this theory is supported by the results of many epidemiological studies that have reported only a minimal effect of such genetic polymorphisms on the risk of

cardiovascular disease [31,33,58-60]. In general it can be concluded that genetic variation may be relevant for the levels of plasma proteins, and the effects of genetic variation on plasma concentrations may be enhanced by environmental factors. However, the relevance of genetic variation to the risk of cardiovascular disease is probably much more limited. Therefore, future *in vivo* and *in vitro* studies on the role of genetic variation are important for a further understanding of the biological mechanisms, but such studies are less relevant to the risk of multicausal diseases with a strong environmental contribution, such as arterial diseases.

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Summary

SUMMARY

Cardiovascular diseases account for approximately 35% of the mortality in the Netherlands. Cardiovascular diseases can be divided into arterial and venous diseases, and atherosclerosis and myocardial infarction are well-known examples of arterial diseases. Cardiovascular diseases are caused by a combination of lifestyle and genetic factors. Elevated plasma levels of the coagulation factor fibrinogen, and of the fibrinolytic factor PAI-1 are associated with an increased risk of (arterial) cardiovascular diseases.

In the promoter regions of the genes encoding the fibrinogen β and the PAI-1 proteins, genetic variations (polymorphisms) are located. Relationships between these polymorphisms and the fibrinogen and PAI-1 plasma protein levels have been described on many occasions, and a relationship with the risk of arterial diseases has also been suggested. Polymorphisms in the promoter regions can affect the habitual fibrinogen β and PAI-1 levels, but they may also affect the response of the genes to the environment. The aim of the studies described in this thesis is to identify the functional polymorphisms in the fibrinogen β and PAI-1 promoters, to determine their effect on the response of the genes to modulating factors, and to elucidate the underlying molecular mechanisms.

Fibrinogen is an acute-phase protein, and plasma fibrinogen levels strongly increase in response to inflammatory stimuli. In addition, genetic variation of the fibrinogen β gene influences plasma fibrinogen levels, and there are indications from population-based studies that inflammation and genetic variation of the fibrinogen β promoter interact. In **chapter 2 and 3**, studies are described that further elucidate the molecular regulation of the fibrinogen β gene, and the interaction of genetic variation with the regulation of transcription. Using luciferase reporter gene assays in hepatoma cells, mutation analysis, and electrophoretic mobility shift assays, we identified a hepatocyte nuclear factor-3 (HNF-3) site located just upstream of a previously identified interleukin-6 (IL6) -responsive sequence in the fibrinogen β promoter. Our results show that this HNF-3 site is essential for a full response of the promoter to IL6. The activity of the CCAAT box/enhancer-binding protein (C/EBP) site (located 18 nucleotides downstream of the HNF-3 site and important to the IL6 response), depends on the integrity of the HNF-3 site and *vice versa*, explaining the necessity of HNF-3 in the IL6 response of the fibrinogen β promoter. Such involvement of HNF-3 in the inflammatory gene response has not been described before, but the collaborative action of these two transcription factors might be more commonly employed as putative HNF-3 sites are also located adjacent to functional C/EBP sites in the fibrinogen α and CRP genes.

In addition, we showed that among six polymorphisms in the fibrinogen β gene (-1420G/A, -933C/T, -854G/A, -455G/A, -249C/T, -148C/T), only the –148C/T variation affected IL6-induced and basal fibrinogen β promoter activity in our assay system. Our results indicate that the -148C/T polymorphism, which is located between the binding sites of HNF-3 and C/EBP, interferes with the interaction between these two transcription factors, which explains its functional effects at the molecular level.

The acute phase reaction is important in many disease processes. Habitual levels of the acute phase proteins fibrinogen, C-reactive protein (CRP) and interleukin-6 (IL6) are associated with an increased risk of cardiovascular disease, but the dynamic variation of plasma levels of acute phase proteins may be of importance as well. The aim of **chapter 4** was to document the variation in fibrinogen, CRP and IL6 levels in response to yellow-fever vaccination (a mild inflammatory stimulus), in 25 healthy individuals. Plasma levels of fibrinogen, CRP and IL6 were determined at baseline and 7 days after vaccination, and polymorphisms in the promoter regions of these genes were determined. After vaccination, fibrinogen levels had changed between -13% and +44%, CRP levels between -88% and +672%, and IL6 levels between -55% and +448%. The change in fibrinogen levels was statistically significant. Genetic variation partly explained the interindividual variation in response, as IL6-174G homozygotes showed a significantly stronger increase in CRP levels than IL6-174C allele carriers. In conclusion, this study suggests that a large interindividual variation exists in the acute phase response to yellow fever vaccination. This study indicates that individuals may be classified as hyper- or hypo-responders, and that genetic variation may influence the responsiveness of an individual. Finally, this study identifies vaccination as a useful model system to investigate the inflammatory response under standardized conditions.

PAI-1 antigen levels are influenced by acquired factors such as body mass index (BMI), and by genetic factors. **Chapter 5 and 6** describe *in vivo* and *in vitro* studies on genetic variation of the PAI-1 promoter, and the relationship with environmental factors, in the first place with BMI. Two polymorphisms have been described in the PAI-1 promoter; the -844A/G and the -675(4G/5G) variations. The 4G allele of the -675(4G/5G) variation has been associated with elevated PAI-1 concentrations and on some occasions with an increased risk of cardiovascular disease. The associated –844A/G variation however, has been investigated less extensively.
In **chapter 5**, the association between the PAI-1 promoter haplotype and PAI-1 antigen levels was investigated in two populations, each including approximately 600 healthy Caucasians. We observed significantly higher PAI-1 concentrations in A-4G homozygotes than in G-5G carriers, but this relationship was restricted to the lowest BMI quartile. In these lean subjects, the PAI-1 concentrations of A-4G/G-5G heterozygotes were reduced to 60-75%,

and the concentrations of G-5G homozygotes to 45-55%, compared to the PAI-1 concentrations of A-4G homozygotes. PAI-1 concentrations increased approximately 4-fold from the lowest to the highest BMI quartile. To assess the direct effect of the PAI-1 promoter haplotype on PAI-1 promoter activity, *in vitro* reporter gene assays were performed in HepG2 and BAEC cells. These assays however, did not indicate a direct effect of the PAI-1 promoter haplotype on promoter activity. Therefore it is not clear whether one of the variations in the promoter region is responsible for the observed effects, or that the functional variation should be found outside of the promoter region of the PAI-1 gene. Concluding, the study described in **chapter 5** suggests that the PAI-1 promoter haplotype and BMI affect PAI-1 concentrations, but that BMI is a stronger determinant than PAI-1 promoter variation.
Chapter 6 is a description of the relationship between PAI-1 antigen levels, several metabolic and inflammatory parameters and several genetic variations, in a population of approximately 600 healthy Caucasian subjects. Positive correlations were present between PAI-1 antigen levels and the plasma levels of several metabolic factors, C-reactive protein, and low physical activity. In addition, the previously reported relationship between PAI-1 antigen levels and the PAI-1 -675(4G/5G) polymorphism was detected also in this population. Finally, a relationship between the insulin receptor substrate-1 (IRS-1) *Gly*972*Arg* polymorphism and PAI-1 antigen levels was observed, which may be explained by the elevated insulin levels associated with the IRS-1 *Gly*972*Arg* polymorphism, which on their turn increase PAI-1 production. In general, this study demonstrates that PAI-1 plasma levels are under control of several environmental and genetic factors.

This thesis provides more information on the relationships between genetic variation of the fibrinogen β and PAI-1 promoters their respective plasma protein levels. In addition, these studies provide further insight into the mechanisms behind these relationships, which contributes to the understanding of mechanisms concerning cardiovascular disease.

Samenvatting

SAMENVATTING

Hart- en vaatziekten veroorzaakten ongeveer 35% van de sterfgevallen in Nederland in 2001, en kunnen ruwweg opgedeeld worden in twee categorieën, de arteriële en de veneuze ziekten. Van de arteriële (=slagaderlijke) ziekten zijn het hartinfarct en atherosclerose de bekendste verschijningsvormen. Hart- en vaatziekten zijn multifactoriële ziekten, en worden veroorzaakt door een combinatie van omgevings- en genetische factoren. Omgevingsfactoren betreffen vaak leefstijlfactoren (rook- en eetgewoonten, lichamelijke activiteit), of de gevolgen hiervan (overgewicht, diabetes). Verscheidene biologische processen in het lichaam kunnen, als ze verstoord zijn, bijdragen aan het ontstaan van hart- en vaatziekten. Voorbeelden zijn bloedstolling, fibrinolyse (de tegenhanger van bloedstolling), ontsteking, de vetstofwisseling en de glucosehuishouding. Genetische factoren kunnen bijdragen aan de kans op hart- en vaatziekten, doordat ze bovengenoemde biologische processen kunnen beïnvloeden. Er bestaan zeldzame genetische afwijkingen met soms zeer ernstige gevolgen voor hart en/of bloedvaten, maar er bestaan ook veelvoorkomende genetische veranderingen met mildere effecten. Over deze laatste categorie gaat dit proefschrift. Deze veranderingen in de genen worden ook wel genetische variaties of polymorfismen genoemd. Polymorfismen leveren een bijdrage aan het totale risico op hart- en vaatziekten van een individu. Polymorfismen komen onder meer voor in de promoter, het deel van het gen dat de genactiviteit onder verschillende omstandigheden reguleert. Het is waarschijnlijk dat polymorfismen in de promoters van genen de reactie van een individu op omgevingsfactoren kan beïnvloeden.

Verhoogde bloedniveaus van de bloedstollingsfactor fibrinogeen of de fibrinolytische factor 'plasminogen activator inhibitor-1' (PAI-1) zijn geassocieerd met een verhoogd risico op arteriële hart- en vaatziekten, daarom staan fibrinogeen en PAI-1 in dit proefschrift centraal.
Fibrinogeen is een stollingsfactor die in de laatste stap van het bloedstollingsproces omgezet wordt in fibrine. Fibrine vormt samen met de bloedplaatjes het bloedstolsel. Fibrinogeen wordt gemaakt in de lever, en het fibrinogeen molecuul bestaat uit twee sets van drie eiwitketens: 2 α, 2 β en 2 γ ketens. Tijdens de synthese van het fibrinogeen molecuul is de aanmaak van de β keten snelheidsbepalend, daarom richtten wij ons in ons onderzoek op de regulatie van het fibrinogeen β gen. Fibrinogeen is een acuut-fase eiwit, wat betekent dat de productie van fibrinogeen sterk stijgt in reactie op een ontstekingsstimulus zoals infectie, verwonding, zware fysieke inspanning, of roken. Tijdens een acuut-fase reactie worden allerlei ontstekingsstoffen gemaakt, onder andere interleukine-6 (IL6). IL6 stimuleert vervolgens de productie van fibrinogeen.
PAI-1 is een remmer van de fibrinolyse, en verhoogde PAI-1 niveaus in het

bloed leiden tot het vertraagd oplossen van bloedstolsels. PAI-1 wordt geproduceerd op verschillende plaatsen in het lichaam, waarvan het endotheel (vaatwand), de lever en het vetweefsel de belangrijkste zijn. PAI-1 bloedniveaus zijn sterk geassocieerd met het metabool syndroom, wat een overkoepelende term is voor een cluster symptomen die vaak samen voorkomen, te weten; overgewicht, verhoogde lipide- en cholesterolniveaus in het bloed, verhoogde bloeddruk, en verhoogde insuline- en glucosespiegels.
In de promoters van zowel het fibrinogeen β als het PAI-1 gen komen polymorfismen voor die in populatiestudies samengaan met verhoogde bloedniveaus van fibrinogeen of PAI-1. Daarnaast is in enkele onderzoeken ook een verband tussen de polymorfismen in de fibrinogeen β en PAI-1 promoters en een verhoogd risico op (arteriële) hart- en vaatziekten gevonden. In ons onderzoek waren wij dus geïnteresseerd in het mogelijke directe effect van deze polymorfismen op fibrinogeen en PAI-1 bloedniveaus, en in de verklarende moleculair-biologische mechanismen.

Tot nu toe zijn in de fibrinogeen β promoter zes polymorfismen beschreven; -1420G/A, -993C/T, -854G/A, -455G/A, -249C/T en -148C/T, en ook de meest voorkomende combinaties (haplotypen) zijn bekend. Het haplotype -1420A, -993T, -455A en -148T heeft een frequentie van 20% in de populatie, en is geassocieerd met verhoogde fibrinogeen niveaus in het bloed.

Hoofdstuk 2 en 3 beschrijven de resultaten van laboratoriumonderzoek die het functionele polymorfisme in dit haplotype identificeren en het biochemische mechanisme ophelderen. Wij hebben met moleculaire technieken verscheidene fibrinogeen β promoter haplotypen (zowel natuurlijk voorkomende als kunstmatige combinaties) gekoppeld aan het luciferase gen. Deze fibrinogeen β promoter-luciferase constructen zijn in HepG2 cellen (een humane levercellijn) getransfecteerd. Deze cellen produceren vervolgens luciferase onder controle van de fibrinogeen β promoter, en de hoeveelheid geproduceerd luciferase is een maat voor de activiteit van de fibrinogeen β promoter. In onze experimenten beïnvloedde alleen het -148C/T polymorfisme de activiteit van de fibrinogeen β promoter, en dit effect was zeer duidelijk in aanwezigheid van de ontstekingsmediator IL6. Deze conclusie heeft gevolgen voor epidemiologische onderzoeken. In epidemiologische studies wordt om historische redenen vaak het –455G/A polymorfisme bepaald. Deze studies betroffen tot nu toe vrijwel altijd Caucasiërs (mensen van West-Europese of West-Aziatische oorsprong). In deze groep bevinden zich de -455G/A en -148C/T polymorfismen altijd op hetzelfde haplotype. Bepaling van het -455G/A of het -148C/T polymorfisme levert dus hetzelfde resultaat op. In andere etnische groepen zijn deze polymorfismen echter niet altijd geassocieerd, en het is inmiddels aangetoond dat in Afro-Amerikanen fibrinogeen bloedniveaus een sterkere relatie vertonen met het -148C/T polymorfisme dan met het -455G/A polymorfisme. Dit ondersteunt onze conclusie dat -148C/T het functionele polymorfisme in de fibrinogeen β

promoter is.
Om het mechanisme van het effect van het -148C/T polymorfisme op de IL6-respons van de fibrinogeen β promoter te begrijpen, hebben we eerst de omringende DNA sequentie nauwkeurig bestudeerd. Naast het -148C/T polymorfisme in het DNA ligt een (al bekende) herkenningsplaats voor de transcriptiefactor CCAAT-box/enhancer-binding protein β (C/EBPβ). C/EBPβ kan door IL6 geactiveerd worden, en brengt vervolgens het IL6 signaal op de fibrinogeen β promoter over, waardoor deze gestimuleerd wordt. Aan de andere kant van het -148C/T polymorfisme bleek een DNA sequentie te liggen die sterk leek op een bindingsplaats voor de leverspecifieke transcriptiefactor hepatocyte-nuclear factor-3 (HNF-3). Naar deze mogelijke HNF-3 site hebben wij verder onderzoek gedaan. Electrophoretic mobility shift assays (Eiwit-DNA bindingsassays) toonden aan dat HNF-3 inderdaad kan binden op deze positie naast het -148C/T polymorfisme. Om het belang van deze HNF-3 site voor de activiteit van de fibrinogeen β promoter te onderzoeken, hebben we transfectie assays gedaan met een fibrinogeen β promoter variant met een gemuteerde HNF-3 site. Mutatie van de HNF-3 site leidde tot een sterk verminderde reactie van de promoter op tot overexpressie gebracht HNF-3, wat aangeeft dat het hier inderdaad een functionele site betreft. Daarnaast bleek de gemuteerde promoter verzwakt te reageren op IL6 en C/EBPβ, de overbrenger van het IL6 signaal. Deze proeven bewezen dat de HNF-3 site belangrijk is voor de reactie van de fibrinogeen β promoter op IL6, waarschijnlijk omdat HNF-3 door binding aan deze site samenwerkt met C/EBP. In de literatuur is HNF-3 beschreven als een 'opener' van het DNA. Het is daarom mogelijk dat in geval van de fibrinogeen β promoter, HNF-3 het DNA toegankelijk maakt voor C/EBPβ, de overbrenger van het IL6 signaal. Een rol voor HNF-3 in de regulatie van genen door IL6 is nog niet eerder beschreven, en wij hebben aanwijzingen dat dit een algemener genregulatie mechanisme zou kunnen zijn. Tenslotte bleek het -148C/T polymorfisme de interactie tussen de HNF-3 site en de C/EBPβ site te beïnvloeden, wat het effect van dit polymorfisme op de IL6-respons van de fibrinogeen β promoter kan verklaren.

Er bestaan aanzienlijke verschillen tussen mensen in de mate waarin ze op specifieke omgevingsfactoren reageren, en het is waarschijnlijk dat genetische variatie in het promoter gebied van genen bijdraagt aan deze interindividuele variatie. Het kan lastig zijn om deze hypothese in een populatie te onderzoeken, omdat het in sommige gevallen onethisch is de onderzoeksgroep bloot te stellen aan de (mogelijk schadelijke) omgevingsfactoren. Dit geldt zeker voor de voor ons onderzoek interessante ontstekingsstimuli, zoals verwonding en infecties. Het is bekend dat sommige vaccins (o.a. het gele koorts vaccin), een milde ontstekingsreactie kunnen oproepen, en daarmee zou vrijwillige vaccinatie dus een ethisch aanvaardbaar acuut-fase-onderzoeksmodel kunnen zijn. In **hoofdstuk 4** hebben we bepaald in hoeverre mensen verschillen in hun reactie op gele koorts vaccinatie, en daarmee onderzocht of vaccinatie een bruikbaar onderzoeksmodel voor ontsteking was. Vervolgens hebben we onderzocht of

genetische variatie de variatie in ontstekingsrespons tussen mensen deels kan verklaren. Bij mensen die werden gevaccineerd tegen gele koorts in verband met een voorgenomen reis naar Azië of Zuid-Amerika, werd bloed afgenomen voorafgaand aan de vaccinatie en 7 dagen daarna. Vervolgens zijn de niveaus van fibrinogeen en van ontstekingsmarker C-reactief proteïne (CRP) bepaald. Tenslotte hebben we de polymorfismen in de fibrinogeen β- en de IL6 -promoter bepaald. De IL6 promoter werd betrokken in het onderzoek omdat IL6 de belangrijkste stimulator van zowel CRP als fibrinogeen is, en omdat er in het verleden relaties zijn gevonden tussen polymorfismen in de IL6 promoter en CRP bloedniveaus. Uit statistische analyse van de resultaten bleek dat er tussen mensen inderdaad een zeer grote variatie in de reactie van fibrinogeen en CRP bloedniveaus op gele koorts vaccinatie bestaat. Daarnaast was er ook een verband tussen genetische variatie en de mate van reactie op de vaccinatie. Genetische variatie in de IL6 promoter bleek gerelateerd aan de mate van stijging van CRP bloedniveaus na gele koorts vaccinatie. Een dergelijke trend bleek er ook te bestaan tussen polymorfismen in de fibrinogeen β promoter en de reactie van fibrinogeen bloedniveaus op vaccinatie. Concluderend geeft onze studie aan dat gele-koorts vaccinatie een bruikbaar model is om de interindividuele variatie in ontstekingsreactie te bestuderen. Daarnaast ondersteunen onze onderzoeksresultaten de hypothese dat genetische variatie in de promoters van genen de genrespons mede bepaalt. Wel moeten we opmerken dat de absolute effecten van de onderzochte polymorfismen op de bloedniveaus vrij klein waren, wat aangeeft dat het belang van de individuele polymorfismen voor het individu waarschijnlijk vrij beperkt zijn.

Verscheidenen populatiestudies hebben aangetoond dat de –675(4G) variant van het -675(4G/5G) polymorfisme in de PAI-1 promoter gerelateerd is aan verhoogde PAI-1 bloedniveaus. Echter, in deze studies is een ander polymorfisme in de PAI-1 promoter (-844A/G) buiten beschouwing gelaten. In onze studies hebben we ook het –844A/G polymorfisme, en de PAI-1 promoter haplotypen in beschouwing genomen (**hoofdstuk 5)**. Hiervoor hebben wij een nieuwe, haplotype-specifieke PCR methode ontwikkeld, waarmee we de PAI-1 promoter haplotypen bepaald hebben in twee onderzoeksgroepen van elk ongeveer 600 gezonde Caucasiërs. Vervolgens hebben we in deze mensen ook de PAI-1 bloedniveaus bepaald. Uit statistische analyse van de resultaten bleek in beide populaties een relatie te bestaan tussen het PAI-1 promoter haplotype en PAI-1 bloedniveaus. Hogere PAI-1 niveaus gaan samen met het -675(4G)/-844A haplotype, en lagere PAI-1 niveaus met het -675(5G)/-844G haplotype. Echter, deze relatie was alleen te detecteren in slanke mensen. Dit is te verklaren uit het feit dat PAI-1 bloedniveaus zeer sterk stijgen met toenemend lichaamsgewicht, en het is waarschijnlijk dat in mensen met overgewicht het effect van het PAI-1 haplotype op PAI-1 bloedniveaus overschaduwd wordt door het sterkere effect van een hoger lichaamsgewicht. Deze bevinding kan het belang van het PAI-1 promoter haplotype voor het totale risico op hart- en vaatziekten ter discussie stellen. Immers, alleen in

slanke mensen lijkt het PAI-1 haplotype een meetbaar effect op PAI-1 levels te hebben, en dit zijn nu juist de mensen die vanwege hun lage lichaamsgewicht sowieso al een lager risico op (arteriële) hart- en vaatziekten hebben. Onze bevinding stemt overeen met die van eerdere onderzoeken, waarin meestal slechts een zwak of geen verband tussen PAI-1 promoter polymorfismen en risico op hart- en vaatziekten gevonden is. Over de rol van de andere twee PAI-1 promoter haplotypen (-675(4G)/-844G en -675(5)/-844A) hebben we geen uitspraak kunnen doen omdat deze twee haplotypen vrij zeldzaam bleken te zijn. Hierdoor was de statistische zekerheid te laag om over de relatie van deze twee haplotypen met PAI-1 bloedniveaus een uitspraak te kunnen doen.
Om toch meer te weten te komen over de (effecten van de) twee zeldzame haplotypes en over de polymorfismen afzonderlijk, hebben we ook PAI-1 promoter-luciferase experimenten gedaan. Hiervoor hebben we HepG2 cellen en BAEC cellen (endotheelcellen geïsoleerd uit kalfsaorta's) gebruikt. In deze experimenten hebben wij geen effect van de -844A/G en -675(4G/5G) polymorfismen, in welke combinatie dan ook, op PAI-1 promoter activiteit gevonden. Dit lijkt verrassend vanwege de aanwezigheid van de relatie tussen PAI-1 promoter haplotypen en PAI-1 bloedniveaus in de onderzoeksgroepen, maar de afwezigheid van een direct effect van de haplotypen op PAI-1 promoter activiteit in de experimenten is verklaarbaar. Mogelijk is een ander polymorfisme dan -675(4G/5G) of -844A/G, gelegen buiten de promoter maar wel samen voorkomend met de promoter polymorfismen, verantwoordelijk voor de effecten op bloedniveaus. Dit veronderstelde polymorfisme zou dus aanwezig zijn in de PAI-1 genen van de mensen in de onderzoeksgroep, maar ontbreekt in de luciferase experimenten die alleen de polymorfismen in de promoter in beschouwing namen. In andere delen van het PAI-1 gen zijn inderdaad polymorfismen aanwezig die (vrijwel) volledig samen voorkomen met de -675(4G/5G) en -844A/G polymorfismen. In het bijzonder de polymorfismen aan het uiteinde van het gen (het zogenaamde 3' deel) kunnen erg interessant zijn, omdat er steeds meer bewijs komt dat behalve de promoter ook het 3' deel van genen bepalend is voor de expressieniveaus van genen. Het is dus mogelijk dat de polymorfismen in het 3' gebied de PAI-1 expressie beïnvloeden.

Hoofdstuk 6 beschrijft de relaties van verscheidene omgevings- en genetische factoren met PAI-1 bloedniveaus in een populatie van 600 gezonde Caucasiërs. Dit hoofdstuk geeft meer achtergrondinformatie, en mogelijk nieuwe aanknopingspunten voor verder onderzoek. In onze onderzoeksgroep bleken PAI-1 plasma niveaus gerelateerd te zijn aan de verscheidene facetten van het metabool syndroom, en aan een verhoogde ontstekingsstatus (gereflecteerd door verhoogde bloedniveaus van de ontstekingsmarker CRP). Daarnaast vonden we verhoogde PAI-1 niveaus in mensen met overgewicht, in mensen die meer dan twee glazen alcohol per dag dronken, in mensen die lichamelijk minder actief zijn, en in niet-rokers (waarschijnlijk omdat de niet-rokers gemiddeld wat zwaarder zijn). Deze bevindingen bevestigen eerder

gerapporteerde resultaten. Wat betreft genetische variatie, vonden we de verwachte relatie tussen het -675(4G/5G) polymorfisme en PAI-1 bloedniveaus, en een relatie tussen het insuline receptor substraat-1 (IRS-1) *Gly*972*Arg* polymorfisme en PAI-1 plasma levels. IRS-1 is een belangrijke schakel in de overdracht van het insuline-signaal, en het is gebleken dat dragers van de IRS-1 972*Arg* variant een verhoogd insuline bloedniveau hebben. Omdat insuline ook een belangrijke stimulator van PAI-1 expressie is, kan de verhoogde insulinespiegel in dragers van de IRS-1 972*Arg* variant het hogere PAI-1 bloedniveau verklaren.

De effecten van genetische variatie in de promoter gebieden van de fibrinogeen β en PAI-1 genen stonden centraal in het onderzoek dat in dit proefschrift beschreven is. Dit proefschrift geeft nieuwe inzichten in de relatie tussen genetische variatie van de fibrinogeen β en PAI-1 promoters en de fibrinogeen en PAI-1 bloedniveaus, en de onderliggende (moleculaire) mechanismen. Vanwege de sterke relatie tussen fibrinogeen en PAI-1 bloedniveaus en hart- en vaatziekten, draagt dit bij aan de basiskennis die noodzakelijk is voor het beter begrijpen van het ontstaan en de ontwikkeling van hart- en vaatziekten.

Nawoord

NAWOORD

Dit proefschrift is het resultaat van vier jaar onderzoek uitgevoerd bij TNO Preventie en Gezondheid in Leiden en bij de sectie Hemostase van het Leids Universitair Medisch Centrum, onder begeleiding van Dr. Moniek de Maat en Dr. Hans Vos. Promoveren doe je niet alleen, dus op deze plaats wil ik graag aandacht geven aan een paar mensen in het bijzonder.

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'Mijn boekje is bijna af….'…… ik beloof dat ik jullie nooit meer zo zal foppen…

Curriculum Vitae

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 12 februari 1975 geboren te Wageningen.

Na het behalen van haar VWO diploma in 1993 aan Scholengemeenschap Durendael in Oisterwijk, ging zij Bioprocestechnologie studeren aan Wageningen Universiteit. Tijdens de studie werden twee afstudeervakken en een stage uitgevoerd. Tijdens het eerste (7-maands) afstudeervak bij de vakgroep Virologie werd een moleculair-biologisch onderzoek gedaan naar een vaccin tegen mond- en klauwzeer, onder begeleiding van Dr. D. Zuidema. Tijdens een tweede (5-maands) afstudeervak bij de vakgroep Communicatie en Innovatie studies werden de dilemma's die leven rond biotechnologie bij dieren in kaart gebracht, met discursieve psychologie als onderzoeksperspectief. Dit onderzoek werd begeleid door Dr. H. F.M te Molder. Tenslotte werd een 5-maands stage uitgevoerd aan the University of British Columbia in Vancouver B.C., Canada, in 'the Laboratory of Marine Virology' onder leiding van Prof. Dr. C.A. Suttle. Tijdens deze stage werd onderzoek gedaan naar de moleculaire biologie en de moleculaire ecologie van mariene virussen.

Na het behalen van het doctoraal examen in september 1999, begon de schrijfster van dit proefschrift in november van datzelfde jaar aan haar promotieonderzoek onder leiding van Dr. M.P.M. de Maat (afdeling Biomedical Research, TNO Preventie en Gezondheid in Leiden) en Dr. H.L. Vos (sectie Hemostase en Trombose onderzoek, afdeling Hematologie van het Leids Universitair Medisch Centrum). De resultaten van dit onderzoek zijn te lezen in dit proefschrift.

Sinds mei 2004 is de schrijfster van dit proefschrift werkzaam als Scientist op de afdeling Up Stream Processing van Crucell in Leiden, waar zij bijdraagt aan de ontwikkeling van vaccins.